8TH INTERNATIONAL CONGRESS ON NITROGEN FIXATION

Knoxville, May 20-26, 1990

FINAL PROGRAM

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THE UNIVERSITY OF TENNESSEE
KNOXVILLE

Division of
Continuing
Education

UT
Conference
Center

May 20, 1990

Dear Attendee,

Welcome to Knoxville and the 8th International Congress on Nitrogen Fixation!

We are happy to have you here and we hope you enjoy your stay.

The following pages in your booklet will provide you with general information, a program schedule, abstracts, helpful phone numbers and special event and drink tickets. The abstracts have been color coded for ease in finding a particular group. Please take a moment to familiarize yourself with the contents of the booklet.

UT Conferences' staff and other UT volunteers will be available to answer your questions and assist you as best we can. (*Look for anyone wearing a "Host" ribbon on their name badge.*)

Again, welcome to Knoxville and the University of Tennessee!

Lea Anne Law
Program Manager

600 Henley St., P.O. Box 2648 Knoxville, Tennessee 37901 (615) 974-0250 FAX: (615) 974-0264

8TH INTERNATIONAL CONGRESS ON NITROGEN FIXATION

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SUNDAY NIGHT

3:00- 8:00	Registration Desk Open - ***Upper Escalator Lobby***
7:00-9:00 pm	Mixer and Official Opening of the Congress - ***Grand Ballroom***

MONDAY

Plenary Lectures - Grand Ballroom

8:00-5:00	Registration Desk Open
8:30- 8:35 am	**Desh Pal Verma** (Chairperson): Opening Remarks
8:35- 9:20	**Barry Smith:** Nitrogenases, old and new
9:20-10:05	**Sharon Long:** Genetics of nodulation; the plant-bacterium interface
10:05-10:35	Morning coffee break
10:35-11:20	**David Layzell:** Oxygen and the regulation of nitrogen fixation in legume nodules
11:20-12:05 pm	**Frans De Bruijn:** Regulation of nitrogen fixation and assimilation genes in the free-living versus symbiotic state
12:05- 2:00	LUNCH - *Exhibit Hall 1*
Noon-2:00	Poster & exhibit set-up - *Exhibit Hall 2*

Session A: Recent advances in the chemistry of nitrogen fixation

2:00- 2:05	**Bill Orme-Johnson** (Chairperson): Opening remarks
2:05- 2:30	**Barbara Burgess:** Characterization of altered forms of the MoFe protein of *Azotobacter vinelandii* nitrogenase
2:30- 2:55	**Vinod Shah:** *In vitro* synthesis of the iron-molybdenum cofactor and its analogs: requirement of a non-*nif* gene product for the synthesis, and properties of dinitrogenases
2:55- 3:20	**Roger Thorneley:** Kinetics and mechanisms of ATP hydrolysis, electron transfers and proton release by *Klebsiella pneumoniae* nitrogenase
3:20- 3:50	**Grand Opening of Exhibits - *Exhibit Hall 2***
3:50- 4:15	**Robert R. Eady:** Mo-independent nitrogenases of *Azotobacter*
4:15- 4:30	**Millie Georgiadis:** Progress on the structure of the nitrogenase iron protein from *Azotobacter vinelandii*
4:30- 4:45	**Jeff Bolin:** Crystallographic studies on the Mo-Fe protein structure: recent results
4:45- 5:10	**Demitri Coucouvans:** Metal clusters
5:10- 6:30	Poster session - ***Exhibit Hall 2***
6:30- 8:15	**DINNER** (*On your own*)

Provocative session - Grand Ballroom

8:15-8:20 pm	**Maurizio Iaccarino** (Chairperson): Opening remarks
8:20- 9:05	**Ton Schoot Uiterkamp:** Nitrogen cycling and human intervention
9:05- 9:50	**Janet Sprent:** Structure, function, and evolution of nitrogen-fixing root nodules: confessions of ignorance

TUESDAY

Session B: Plant-microbe interaction - Grand Ballroom
Exhibits open all day - *Exhibit Hall 2*

8:30-8:35 am	**Frank Dazzo** (Chairperson): Opening remarks
8:35- 9:00	**Jean Dénarié:** *Rhizobium meliloti* extracellular nod signals
9:00- 9:25	**Jan Kijne:** Surface interactions between rhizobia and legume root hairs
9:25- 9:50	**Saleela Philip-Hollingsworth:** Isolation, characterization and structural elucidation of a "nod-signal" excreted by *Rhizobium trifolii* ANU843 which induces root hair branching and nodule-like primordia in axenic white clover seedlings
9:50-10:20	Morning coffee break - ***Exhibit Hall & various stations***
10:20-10:45	**Allan Downie:** Exported proteins encoded by *nod* genes from *Rhizobium leguminosarum* biovar *viciae*
10:45-11:10	**David Day:** Nutrient exchange across the peribacteroid membrane
11:10-11:35	**Nick Brewin:** Immunological dissection of the plant-microbial interface in pea nodules
11:35- 1:30 pm	**LUNCH** and poster viewing - ***Exhibit Hall 1 & 2***
	Discussion group on *Medicago*

Session C: Limiting factors of nitrogen fixation

1:30- 1:35	**Rafael Palacios** (Chairperson): Opening remarks
1:35- 2:00	**John Witty:** Oxygen diffusion and regulation of nitrogenase/hydrogenase in nodules
2:00- 2:25	**Shigeyuki Tajima:** Metabolism of C_4-dicarboxylates and amino acids in soybean nodule bacteroids, and their role to energy supply to nitrogenase
2:25- 2:50	**Hauke Hennecke:** A genetic approach to analyze the critical role of oxygen in bacteroid metabolism
2:50- 3:15	**Robert Maier:** Molecular analysis of terminal oxidases in electron transport pathways of *Bradyrhizobium japonicum* and *Azotobacter vinelandii*
3:15- 3:45	Afternoon coffee break - ***Exhibit Hall & various stations***
3:45- 4:10	**Fraser Bergersen:** Measurement of N_2 fixation by ^{15}N natural abundance, in the management of legume crops: roles and precautions
4:10- 4:35	**Tom LaRue:** Map positions of *sym* genes involved in nodulation and nitrogen fixation in *Pisum sativum*
4:35- 5:00	**Donald Phillips:** Host legume control of nodulation and other rhizosphere events through release of flavanoids

Harold Evans lecture - Grand Ballroom

5:00- 5:05	**Herman Bothe** (Chairperson)
5:05- 5:40	**Daniel Arp:** H_2 cycling in N_2 fixation: past, present and future outlook
6:30-10:30pm	**Southern Heritage Showcase at World's Fair Park**
	6:30-8:15 BBQ Dinner Served
	7:00-10:00 Sunsphere Observation Deck Open
	7:30-10:30 Entertainment Showcase - *Tennessee Amphitheater*

WEDNESDAY

Excursions or free for small meetings

1:00-6:00 pm Poster viewing and Exhibits open - ***Exhibit Hall 2***

Wednesday night program:

Burning issue-poster discussion workshop I - Grand Ballroom

8:00- 9:00 **Gary Stacey and Ben Lugtenberg** (Conveners):
Issue 1: What is the biochemical function of *nod*, *fix* and *nif* genes?

9:00-10:00 **Bill Newton** (Convener):
Issue 2: What are the complexities of the substrate reactions and energetics of nitrogenase?

THURSDAY

Exhibits open all day - ***Exhibit Hall 2***

Session D: Nitrogen fixation and the environment - Grand Ballroom

8:15-8:20 am **Sergey Shestakov** (Chairperson): Opening remarks
8:20-8:45 **Peter Bottomley:** Ecology of rhizobia
8:45-9:10 **Walter Zumft:** Molecular analysis of the denitrification system of pseudomonads
9:10-9:35 **Alan Hooper:** Biochemistry of nitrification
9:35-10:00 **Steve Zinder:** Nitrogen fixation in the methanogenic archaebacterium *Methanosarcina barkeri* 227
10:00-10:30 Morning coffee break - ***Exhibit Hall 2 & various stations***

Mini-symposium on the release of genetically engineered organisms into the environment:
(supported in part by the OECD Project on Biological Resource Management)

10:30-10:40 **S. Shantharam** (Chairperson): Opening remarks
10:40-11:10 **Jim Tiedje** (USA-view): GEM regulation
11:10-11:20 **Ton Schoot Uiterkamp** (Europe-view): GEM regulation
11:30-11:45 **Clive Ronson:** Field release of genetically engineered *Rhizobium meliloti* and *Bradyrhizobium japonicum* strains.
11:45-12:15 pm **Phil Sayre:** Assessment of genetically engineered microorganisms under the toxic substances control act: Considerations prior to small scale field release.
12:15-12:30 General discussion
12:30- 1:30 **LUNCH** and poster viewing - ***Exhibit Halls 1 & 2***

Session E: Bacterial systems - Grand Ballroom

1:30- 1:35 pm **Alf Pühler** (Chairperson): Opening remarks
1:35- 2:00 **Eva Kondorosi:** *Rhizobium meliloti* nodulation genes and their regulation
2:00- 2:25 **Martin Buck:** Regulation of *nif* gene expression in *K. pneumoniae*
2:25- 2:50 **Gary Ditta:** Genes required for oxygen regulation in *Rhizobium meliloti*
2:50- 3:15 **Eduardo Santero:** Mechanism of transcription from *nif* promoters: involvement of integration host factor (IHF)

3:15- 3:40	**Werner Klipp:** Organization and regulation of nitrogen fixation genes of *Rhodobacter capsulatus.*
3:40- 4:10	Afternoon coffee break - ***Exhibit Hall 2 & various stations***

Session F: Nitrogen fixation in agriculture and industry - Grand Ballroom

4:10- 4:15pm	**Ben Bohlool** (Chairperson): Opening remarks
4:15- 4:40	**Alan Paau:** Improvement of inoculant efficacy by strain improvement and formulation manipulations
4:40- 5:05	**K. Mulongoy:** Biofertilizer value of nitrogen-fixing systems in tropical agrosystems
5:05- 5:30	**Jos Vanderleyden:** *Azospirillum* associations
5:30- 5:55	**Maurice Lalonde:** The genetics of actinorhizal *Alnus*
5:55- 6:15	**J. K. Ladha:** Recent studies on stem nodulating legume-*Rhizobium* symbiosis
6:20- 8:15	Poster viewing - ***Exhibit Hall 2***
	DINNER on your own

Thursday night

Burning issue-poster discussion workshop II - Grand Ballroom

8:15- 9:15pm	**Peter Gresshoff and Champa Sengupta-Gopalan** (Conveners): Issue 3: How can we couple the genetics and physiology of nodule function?
9:15-10:15	**Joseph Thomas and Dietrich Werner** (Conveners): Issue 4: What are the applications of our research to ecology and agriculture worldwide?

FRIDAY

Exhibits & Posters - Open till Noon

Session G: Plant function - Grand Ballroom

8:30- 8:35am	**Julie Cullimore** (Chairperson): Opening remarks
8:35- 9:00	**Dave Emerich:** Protein phosphorylation and protein synthesis in *Bradyrhizobium* bacteroids
9:00- 9:25	**Kjeld Marcker:** *Cis* and *trans* regulatory elements involved in the activation of nodule specific genes
9:25- 9:50	**Carroll Vance:** Alfalfa root nodule aspartate aminotransferase (AAT): biochemical importance and genetic control
9:50-10:20	Morning coffee break - ***Exhibit Hall 2 & various stations***
10:20-10:55	**Champa Sengupta-Gopalan:** Regulation of host gene expression during nodule development in soybeans
10:55-11:20	**Ton Bisseling:** Early nodulin genes and infection
11:20-1:20pm	**LUNCH** (oxygen barrier discussion group) - ***Exhibit Hall 1***
Noon	**Exhibits & Posters Close**

Session H: Nitrogen fixation and evolution - Grand Ballroom

1:20-1:25pm	**Barry Rolfe** (*Chairperson*): Opening remarks
1:25- 1:50	**Beth Mullin:** *Frankia* nodulation
1:50- 2:15	**Paul Bishop:** Genetics of alternative nitrogen fixation systems in *Azotobacter vinelandii*
2:15- 2:40	**Bob Haselkorn:** Nitrogen fixation in filamentous cyanobacteria
2:40- 3:05	**Dennis Dean:** Biochemical genetic analysis of the nitrogenase MoFe protein
3:05- 3:35	Afternoon coffee break - ***Exhibit Hall 2 & various stations***
3:35- 4:00	**Jiann-Shin Chen:** Nitrogen fixation genes in *Clostridium pasteurianum*
4:00- 4:25	**Gary Roberts:** The genetics and biochemistry of the reversible ADP-ribosylation systems of *Rhodospirillum rubrum* and *Azospirillum lipoferum*
4:25- 4:50	**Ralph Hardy:** The first photosynthetic nitrogen-fixing *Rhizobium*: characteristics
4:50- 5:15	**Ted Cocking:** Nodulation of non-legumes by rhizobia
5:20- 6:00	**Bob Burris and Wolfgang Bauer:** Congress Summary

Friday evening - Exhibit Hall 1

7:00-Midnight	**CONGRESS BANQUET** *featuring the* Hector Quirko Band
7:00	Reception
7:45	Banquet and Awards
9:15	Hector Quirko Band

SATURDAY

Departure

CONGRESS INFORMATION - Hotline 974-0266

The hotline will be operated from 8:00 am - 5:00 pm Monday-Friday.

Registration & Information: Registration desks will be located in the upper escalator lobby of the Holiday Inn World's Fair. Hours are as follows:

Sunday	3:00 pm - 8:00 pm
Monday	8:00 am - 5:00 pm
Tuesday	8:00 am - 5:00 pm
Wednesday	**Closed**
Thursday	8:00 am - 5:00 pm
Friday	8:00 am - 5:00 pm

Name Badges: Please be sure to wear your name badge at all times. This is your entry into all the sessions.

Dorm Residents: There is 24 hr. check-in for those staying at the dorm beginning on Saturday. Parking is available in Lot N-4 across the street from Clement Hall. Parking permits are required. For those who purchased them in advance, you can pick it up when you check into the dorm. Please place it in the windshield of your automobile. Anyone else needing a parking permit will have to go to the Safety & Security Bldg. at 1115 UT Drive between the hours of 8:00 am - 4:00 pm, Monday - Friday to purchase one.

Participants List: A list of participants is included in your registration material. Addendums will be available at the registration desk as the need arises.

Sessions: A program schedule follows. In general, all speaker sessions are held in the Grand Ballroom and Poster Sessions in Exhibit Hall 2.

Exhibits/Poster Sessions: Exhibitors and the Poster Sessions will be held in the Exhibit Hall 2. The Grand Opening of the Exhibits is scheduled for the afternoon break on Monday. If you need additional information about Poster presentations, please check at the SPEAKER/POSTER DESK in the downstair's escalator lobby of the Convention Center.

Spouses Room: Meeting Room 5 is the gathering place for spouses of Congress attendees. Please come to relax and meet others. More information on tours is available at the TOUR DESK.

Speaker Prep and Press Room: Meeting Rooms 3A & 3B are set aside for meeting with the press, interviews and Speaker's preparation of presentations. A slide projector will be available in the room. Carousel trays can be checked out and then returned to the SPEAKER/POSTER desk in the lower escalator lobby.

Special Event Tickets: Included in your booklet are special event and drink tickets for the Sunday night reception, Tuesday evening Southern Heritage Fair and Friday Closing Banquet. Please be sure to wear your name badge and bring these tickets with you, as they will be collected at the door and inside.

Tour/Travel Desk: A tour/travel desk will be operated on Monday & Tuesday and Thursday & Friday from 8:30 am - 5:00 pm. Information on tours and companion tours will be provided by MEDIA CONCEPTS TRAVEL. Reservations for the Wednesday tours can be made on Monday and Tuesday as space permits. DELTA AIRLINES will operate the desk on Thursday and Friday to assist with return flights. Please stop by for more information.

Airport Shuttle: If you would like to obtain return transportation to the airport, please sign up at the TOUR/TRAVEL DESK. Space is limited and will be reserved on a first come-first serve basis, as long as space lasts. YOU MUST MAKE A RESERVATION IN ADVANCE TO SECURE A SEAT ON THE RETURN SHUTTLE. Return shuttles to the airport will operate on the hour on Friday 5/25 from 11:30 am-6:30 pm and on Saturday, 5/26 from 5:30 am - 6:00 pm.

Mini-Bank: First American Bank will operate a special "bank" on Monday and Tuesday from 9:00 am - 2:00 pm in the Upper escalator lobby. Check cashing services and credit card cash advances will be available. Proper identification is required.

Hess's Department Store: Wednesday, May 23rdHess's invites you to join them for a day filled with musical entertainment, informal modeling, cooking demonstrations, and more! Plus, take advantage of our President's letter and receive a 10% discount on any 1 item of your choice. You can pick up a coupon in the Registration area in the upper escalator lobby. Located across the street from the Convention Center.

Restaurant & City Information: A brochure will be available at the KNOXVILLE INFORMATION DESK located in the upper escalator lobby listing area restaurants and other attractions. Do stop by!

Luncheons: Luncheon buffets are provided through your registration fees on Monday, Tuesday, Thursday and Friday in Exhibit Hall 1. Lunch is scheduled for 1 hr.-55 min. However, on Thurs., only 1 hr. is allotted. Please feel free to enter the serving lines at any time during the first 45 minutes of service.

Refreshments: Participants will find refreshment tables located in the Exhibit Hall 2, as well outside the Ballroom and Exhibit Hall promenade. *Southern specialties available at the tables include:*

Monday morning - *Sawmill gravy & biscuits*. Lumberjacks packed salted fat meat - "fat back" - into the woods as one of their major staples. With a little water and flour added to the fireside frying pan, a thick and tasty gravy was produced. Today, milk versus creek water, is used.

Monday afternoon - *Pralines*. First mentioned in print in 1727, the praline was named for French diplomat Cesar du Plessis-Praslin. Originally made with almonds and sugar, American Creoles of the Deep South substituted the plentiful pecan for the almonds. Heralded by New Orleans, Louisiana as its original, the praline is a favorite among all Southerns.

Tuesday morning - *Apple fritters*. Popular in the South since the middle of the 18th century, the fritter can be made of peach, blueberry, banana or other flavors. A slightly sweet batter is made into which the fruit is folded, then it is dropped by spoonfuls into hot fat, deep-fried and may be sprinkled with powdered sugar. A Southern tradition!

Tuesday afternoon - ***Funnel Cake*** demonstration. Introduced into the South by the Pennsylvania Dutch, this fried batter treat has become a staple in Knoxville since the 1982 World's Fair.

Wednesday evening - ***Goo Goo Clusters.*** In 1921, Nashville, Tennessee's Standard Candy Company made the first Goo Goo Cluster in a copper kettle. Made of luscious caramel, creamy marshmallow, whole roasted peanuts and pure milk chocolate, this Tennessee candy is a real treat!

Thursday afternoon - ***Butter Pecan Ice Cream.*** Knoxville's Mayfield Dairy has provided the region with dairy products for decades. A Southern favorite is Butter Pecan.

Friday afternoon - ***Turtle Pie.*** Pie made with chocolate and caramel and laced with Jack Daniels, Tennessee's famous sour mash whiskey.

SPECIAL EVENTS

Sunday, May 20 - Opening Reception 7:00 - 9:00 pm - *Grand Ballroom* - Requires Reception Ticket & 2 drink tickets.
Welcome by various University and local officials with refreshments and entertainment provided by the Jack Daniels Barrel House Gang. Formed in the early 1900's to entertain groups touring the famous Tennessee distillery, the band is composed entirely of Jack Daniels employees. Current members are:

William Grogan - electric bass
Junior Donegan - rhythm guitar
Ray Rogers - rhythm guitar & leader singer
Herman Blankenship - fiddle
Leonard "Grandpa" Grogan - banjo
Gary Rogers - steel guitar

Tuesday, May 22 - Southern Heritage Showcase 6:30 - 10:30 p.m. - *World's Fair Park* - Requires Southern Heritage Showcase ticket.
A detailed description of the Fair follows along with a map and list of craftspersons. Please look this over to familiarize yourself with the schedule of events and activities available.

Friday, May 25 - Closing Banquet and Dance 7:00 - midnight - *Exhibit Hall 1* - Requires Banquet and two drink tickets.
Prior to the banquet, there will be a social hour with complimentary beer and wine (in exchange for the drink tickets included in your packet). Mixed drinks and aperitifs may be purchased at the Cash Bars. A seven course meal will be complimented with a red and white wine. Additional bottles may be purchased at the cash bars. Entertainment, provided by the Hector Qirko Band, will follow the dinner.

HELPFUL PHONE NUMBERS

Medical Assistance - Call the following number for non-emergency illness and identify yourself as attending the Nitrogen Fixation Congress. University of Tennessee Physicians Referral 544-9640. Hours are 7:00 am - 7:00 pm.

AREA HOSPITALS

UT Medical Center 544-9000

Ft Sanders Regional Medical Center 541-1111

Baptist Hospital 632-5011

St. Mary's Medical Center 971-6011

Children's Hospital 546-7711

Park West Hospital 693-5151

EMERGENCY TELEPHONE NUMBER - Fire, Police, Ambulance, etc. 911

Knoxville Police Department 521-1200

Knox County Sheriff 521-2432

Rural Metro Ambulance Service 588-2110

Poison Control Center 544-9400

Knoxville Rescue Squad 546-4821

University of Tennessee Police Dept. 974-3111

CABS/TAXIS

ABC Airport Taxi 970-4545

Courtesy Cab 522-4114

Yellow Cab 523-5151

Big Orange Cab Co. 523-1127

Star Cab Co. 524-8311

AREA LAUNDRY & DRY CLEANING SERVICE

Sanitary Laundry & Dry Cleaners 522-0753

Village Cleaners 525-7471

Hand-Craft Cleaners 522-0211

SOUTHERN HERITAGE SHOWCASE

The Southern Heritage Showcase on Tuesday, May 22, has been designed and planned specifically for the 8th International Congress on Nitrogen Fixation. The East Tennessee Valley is filled with a rich and colorful heritage. Tonight's festivities will provide you with a sampling of the diversity of the peoples, music and talents this area has to offer.

Considerable logistical planning has been done to provide you with a safe, leisurely and entertaining event. To insure your full enjoyment, please consider the following suggestions:

1. **The event begins at 6:30 p.m.** Caterers, craftspeople, and musicians will be in the park in the day preparing for the evening showcase. Please allow them to set-up according to schedule by waiting until the designated start time of 6:30 p.m. before entering the festivity areas.

2. **Don't forget your name badge and meal ticket.** You must provide these items upon entering the barbecue area. This requirement is part of the security system for the event and we will regret any inconvenience to those attendees that have to return to housing areas to retrieve their ticket and/or badge.

3. In exchange for your meal ticket, you will receive an "official" mug and plate. These Congress mementos will allow you to replenish your food and drink throughout the evening.

4. You may enter the food service line anytime between 6:30-8:00 p.m. Please feel free to enjoy the park and crafts before, during, or after your meal. Should you choose to dine outside the tent area, please remember to dispose of throwaways in the trash receptacles. The beverage refill area located near the Clinch Bridge will be open until 10:30 p.m. for your enjoyment.

5. The crafts people and artisans were hand-picked for the event and their participation is voluntary. They are excited to have been selected to show and demonstrate their work to an international group. You are encouraged to ask questions and to linger in the crafts area. These East Tennesseans will be delighted!

6. The Sunsphere, the symbol of the 1982 World's Fair, will open at 7:00 p.m. to provide you with a panoramic view of metropolitan Knoxville and the surrounding Cumberland and Smoky Mountains. Elevator hosts will provide rides to observation deck until 10:00 p.m. Please remember to discard any throw-aways in the trash receptacles.

7. The Entertainment Showcase begins at 7:30 p.m. with continuous music until approximately 10:30 p.m. Please feel free to come and go from this area as you please. This will ensure that you take advantage of all aspects of the evening events.

8. On-site UT Conference Personnel can be identified by their name badges. If you have questions or need directions, please feel free to ask them or stop by the Information Desk in the barbecue area.

9. The City of Knoxville does not allow open containers of alcoholic beverages on the street. Special permission has been received from the city to serve alcoholic beverages at this event. For your own interests, please do not carry alcoholic beverages out of the World's Fair Park area.

10. **Enjoy the evening! It's been planned for you!**

6:30 p.m. World's Fair Park opens for festivities. Meal served through 8:15 p.m.

6:45 p.m. Strolling musicians entertain in the food service and crafts area through 7:30 p.m.

7:00 p.m. Sunsphere observation decks open. Elevators run through 10:00 p.m.

7:30 p.m. **Entertainment Showcase - *Tennessee Amphitheater***

Strange Company
Nancy Brennan Strange
Don S. Cassell, Jr.
Steve Kaufman

Storytelling
Nancy Brennan Strange

Maple on the Hill
Ban Pedigo
Doug Barron
Ed Cullis
Bobby Martin
Don Cassell
Steve and Marty Kaufman

10:00 p.m. Sunsphere Closes

10:30 p.m. Approximate end of concert

8th International Congress on Nitrogen Fixation

SOUTHERN HERITAGE SHOWCASE
World's Fair Park
Tuesday, May 22, 1990

Awning/ARTISAN	Craft
A 1 SCOONOVER, DEBORAH	Contemporary Stained Glass Items
A 2 BLACKWELL, ROBERT	Handcrafted Briar Smoking Pipes
A 3 EDENS, ELIZABETH	Handspun Wool: Woven Rugs, Bags, Linens
A 4 DEFRIESE, RUTH	Contemporary Handmade Cloth Dolls
B 5 LAMPERT, CHARLES E.	Handmade Kitchen, Carving, & Pocket Knives
B 6 DOTSON, KARAN	Stained Glass Kaleidoscopes & Panels
B 7 NOGGLE, KAREN	Contemporary Handcrafted Leather Goods
B 8 HULTQUIST, KAY	Traditional Appalachian Woven Coverlets
C 9 BARKER, DOROTHY	Pit-Fired Earthenware Pottery
C10 WALSH, ELMER	Blacksmithing
C11 RABY, EARL	Contemporary Wooden Toys & Decorative Items
C12 RABY, SARAH	Contemporary Cloth Dolls & Applique
D13 HENRY, BILL	Whittling, Miniature Tools & Implements
D14 NORRELL, LEILANI	Handwoven Wall Hangings, Rugs, & Linens
D15 CHESNEY, RALPH	Traditional White Oak Baskets
D16 BUTCHER, JESSE	Traditional White Oak Baskets
E17 HURST, ANN STOVER	Brooms & Mops Crafted of Woodland Materials
E18 EVANS, GAIL	Handmade Cornshuck Dolls & Flowers
E19 YARNELL, DAVID	Contemporary Wooden Toys & Decorative Items
E20 SMITH, PAT	Contemporary "Wearable Art" Clothing

Approximate Site Scale: 1" = 16'
(Tent & Awnings not to scale)
Tent: 40' x 120'
Awnings: 20' x 20'

INDEX TO ABSTRACTS

Catagories:	Abstract nrs:	Code:
Session L: Speaker abstracts	L-01 - L-57	White
Session A: Recent advances in the chemistry of nitrogen fixation		Blue
- Chemistry	A-01 - A-23	
Session B: Plant-microbe interaction		Golden Rod
- Flavanoids and rhizosphere exudates	B-01 - B-19	
- LPS/ CPS/ EPS/ glucans	B-20 - B-35	
- Miscellaneous	B-35 - B-39	
Session C: Limiting factors of nitrogen fixation		Green
- Hydrogenase/ H_2	C-01 - C-14	
- Nodule and bacteroid biochemistry	C-15 - C-31	
- Miscellaneous	C-32 - C-35	
Session D: Nitrogen fixation and the environment		Yellow
- Salinity/ acidity	D-01 - D-11	
- Competition	D-12 - D-22	
- Miscellaneous	D-23 - D-54	
Session E: Bacterial systems		Pink
- *Rhizobium* genetics	E-01 - E-30	
- *Bradyrhizobium* genetics	E-31 - E-49	
- *(Brady)rhizobium* physiology/ biochemistry	E-50 - E-63	
- Other bacteria	E-64 - E-110	
Session F: Nitrogen fixation in agriculture and industry		Tan
- Inoculation/ pesticides/ plant growth promotion	F-01 - F-40	
Session G: Plant function		Buff
- Ultrastructure/ immunolocalization	G-01 - G-09	
- Nodulins	G-10 - G-27	
- Nodule physiology	G-28 - G-38	
- Lectins	G-39 - G-42	
- Miscellaneous	G-43 - G-59	
Session H: Nitrogen fixation and evolution		Orange
- Alternative nitrogenase	H-01 - H-06	
- Evolution	H-07 - H-14	
- Nodulation of non-legumes	H-15 - H-19	

SESSION L

Speaker abstracts
L-01 - L-57

L-01

Nitrogenases, Old and New.
Barry E. Smith. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

I shall attempt to set the scene for the afternoon session by reviewing important aspects of progress made since the last Congress in Cologne in 1988. In particular, I shall address the questions of active site structure and biosynthesis, and the mechanisms and energetics of substrate reduction.

What are the new problems posed by the discoveries of the vanadium- and iron-only nitrogenases? How do these help us understand molybdenum nitrogenase? What are the useful approaches to these problems?

350
460

Chapter 2 Form + Function:

→ redundant mechanisms.

3 themes.

phenotypic var. in diff. plant
dissimilar genes have similar functions
multiple copies.

mod P/Q: duplicate copies - 2nd copy on other symplasmata

- electrophysiology - single cell approach / neurobiology of R.M.

- SymM is induced by plant signal (unknown)
  - ↓ nodD like → effects nodD3
  - ↓ controls exoP

± 4 hrs in nodbox wipes out activity, but + 10 does increase.

L-03

Nodule Development and Metabolism: Regulation of Oxygen or by Oxygen?

David B. Layzell
Biology Department, Queen's University,
Kingston, Ont. Canada K7L 3N6

Good evidence now exists that carbohydrate metabolism and N_2 fixation in legume nodules is O_2 limited at all times, and especially under adverse environmental or physiological conditions. A variable barrier to oxygen diffusion in the nodule cortex seems to be the primary factor responsible for maintaining the O_2 limited condition. In this talk, I will begin by summarizing briefly our current understanding of how legume nodules regulate their resistance to O_2 diffusion and then propose a new model for the structure and mechanism of diffusion barrier control. The remainder of the talk will focus on the implications of O_2 regulation with respect to gene expression during nodule development, electron allocation to N_2 fixation, the pathways of C and N metabolism in plant and bacterial fractions and the potential for increasing N_2 fixation in legumes or transferring the ability for N_2 fixation to other, non-leguminous plants.

L-04

Regulation of nitrogen fixation - and assimilation genes in the free-living versus symbiotic state.

Frans J. de Bruijn. Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, F.R.G;
From 7/90: MSU-DOE Plant Research Laboratory and Department of Microbiology, Michigan State University, E. Lansing, Mich. 48824, U.S.A.

Nitrogen fixation is an extremely energy intensive process and the nitrogenase enzyme complex very sensitive to oxygen. Therefore it is not surprising that in most nitrogen-fixing organisms analysed thus far the nitrogen fixation (nif/fix) genes are tightly regulated in response to the concentration of combined nitrogen sources and/or oxygen concentration in the cell. The signal transduction pathways and regulatory circuitries involved in nitrogen- and oxygen control have been subject of intensive studies, both in free-living as well as symbiotic nitrogen fixing organisms. These studies have revealed the existence of common regulatory elements but have also uncovered significant differences, which will be reviewed. An emphasis will be placed on the Klebsiella pneumoniae, Rhizobium meliloti, Bradyrhizobium japonicum and Azorhizobium caulinodans systems. The latter, A. caulinodans, represents a particularly useful organism for these type of regulation studies, since it is capable of dinitrogen dependent (diazotrophic) grown in the free-living state, as well as fixing nitrogen in stem- and root nodules on its host, the tropical legume Sesbania rostrata. This makes a direct comparison of nif/fix gene regulation in these two different physiological state possible.

In strictly free-living diazotrophs, the production of ammonia by nitrogenase is coupled to its assimilation mainly via the glutamine synthetase (GS) - glutamate synthase (GOGAT) cycle and the corresponding genes (gln, glt) are often coordinately regulated. In the case of rhizobia, while in the free-living state ammonium is assimilated also primarily by the GS-GOGAT cycle, in the symbiotic state (bacteroid) nitrogen fixation appears to be uncoupled from assimilation, which is carried out by plant GS in the cytoplasm. Three distinct GS enzymes and corresponding genes (glnA, glnII, glnT) have been identified in rhizobia, which are differentially regulated. Their role in the free-living and symbiotic state will be examined and the possible differences in regulatory circuitries controlling nif/fix and gln/glt will be reviewed.

L-05

Characterization of altered forms of the MoFe protein of *Azotobacter vinelandii* nitrogenase.

B. Burgess.
Department of Molecular Biology and Biochemistry,
University of California, Irvine, CA 92717.

In order to investigate the structural organization and function of the MoFe protein of nitrogenase, we are purifying and characterizing altered forms of the MoFe protein of *Azotobacter vinelandii* nitrogenase. These forms are synthesized by specifically constructed mutant strains which contain defined deletions in specific *nif* genes. These mutants fall into two categories, those which have alterations in FeMo cofactor biosynthesis (Δ*nif*H, Δ*nif*E, Δ*nif*B, and Δ*nif*V) and those which produce partially active MoFe proteins that cannot be activated by addition of isolated FeMo cofactor (Δ*nif*Z, Δ*nif*W). Although different mutants of the former group (e.g. Δ*nif*H, Δ*nif*E) appear to have very similar phenotypes with respect to activation by FeMo cofactor in cell-free extracts, our data show that they do not contain the same forms of the MoFe protein. These proteins will be compared with respect to their behavior on anaerobic native gel electrophoresis, their behavior during protein purification and their abilities to be restored to an active state before and after purification.

L-06

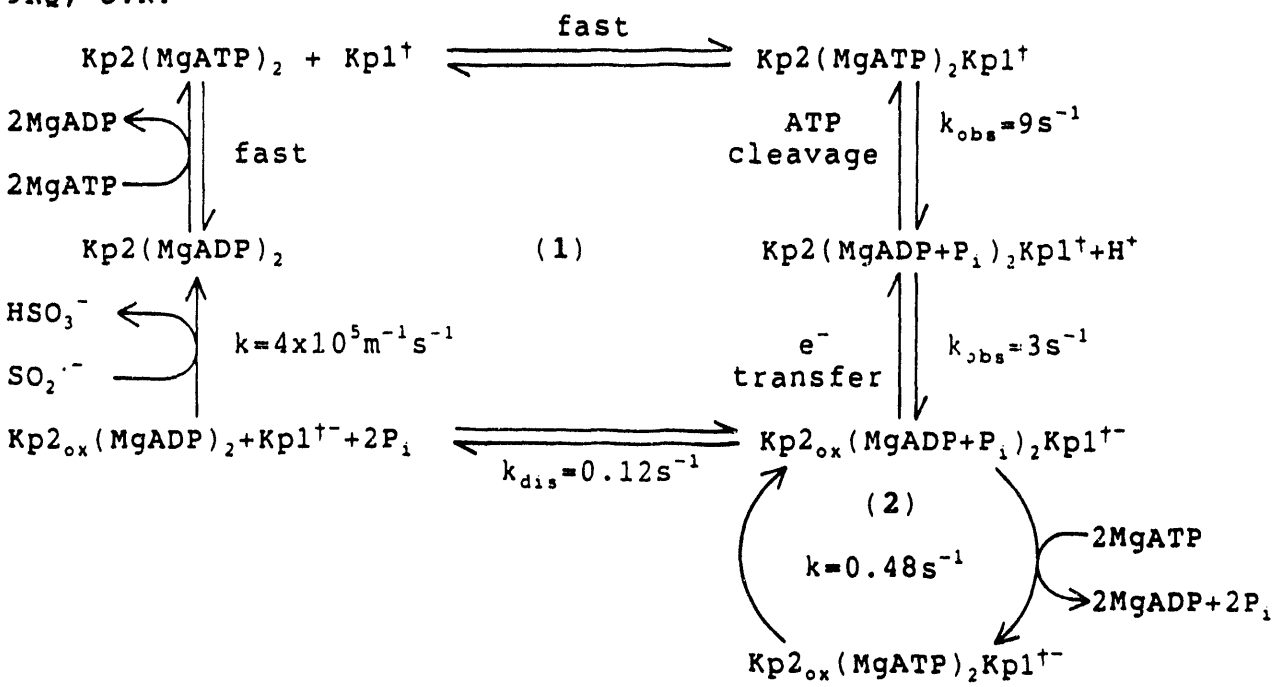
In vitro synthesis of the iron-molybdenum cofactor and its analogs: requirement of non-*nif* gene product for the synthesis, and properties of dinitrogenases. V.K. Shah, M.S. Madden and P.W. Ludden. Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA.

Homocitrate has been shown to be an integral constituent of the iron-molybdenum cofactor (FeMo-co) of dinitrogenase. The *in vitro* FeMo-co synthesis system allows the incorporation of homocitrate and its analogs into the FeMo-co and its incorporation into apo-dinitrogenase. Substrate (N_2, C_2H_2, HCN or H^+) reduction properties and inhibitor (CO or COS) susceptibilities of dinitrogenase are dictated by homocitrate or its analog (e.g. citrate, homoisocitrate, isocitrate, 1-OH citrate and 1-F-homocitrate) used during the FeMo-co systhesis. Dinitrogenase activated with homocitrate-FeMo-co reduces dinitrogen, acetylene, cyanide and protons efficiently, while dinitrogenase activated with 1-OH acids (homoisocitrate, isocitrate and 1-OH citrate) did not reduce dinitrogen or acetylene, but showed intermediate cyanide reduction activities and high proton reduction activities. On the other hand, citrate activated dinitrogenase showed very low dinitrogen and cyanide reduction activities, but intermediate acetylene and proton reduction activities. Dinitrogenases activated *in vitro* using diastereomers of 1-F-homocitrate differ significantly in their substrate reduction properties and inhibitor susceptibilities, thus suggesting that the stereochemistry of C-1 of homocitrate is critical in determining the interaction of FeMo-co with substrates and inhibitors. Substrate specificities and inhibitor susceptibilities of dinitrogenases activated *in vitro* with homocitrate and its analogs will be discussed.

Purification of the *nif* gene products involved in the synthesis of FeMo-co and its insertion into apo-dinitrogenase has allowed us to discover the requirement of a component not suspected to be involved in the mechanism.

L-07

Kinetics and mechanisms of ATP hydrolysis, electron transfers and proton release by Klebsiella pneumoniae nitrogenase.
Roger N.F. Thorneley. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.



The Scheme shows catalytic cycles for reductant-dependent (1) and reductant-independent ATPase activities (2). Kp2 signifies the Fe-protein with two MgATP or MgADP binding sites. $Kp1^{\dagger}$ represents one of two independently functioning halves of the tetrameric Kp1, MoFe-protein. Each $Kp1^{\dagger}$ is assumed to contain one Mo substrate binding site and one Kp2 binding site. The rate constants have been determined by stopped-flow spectrophotometry, stopped-flow calorimetry and steady-state H_2 and P_i assays at 6°C, pH 7.0. The ATP:2e ratio under these conditions was ca. 20:1. Mechanistic conclusions are:-

(1) On enzyme MgATP cleavage to yield bound MgADP+P_i is endothermic, involves concomitant release of one proton and precedes electron transfer from Kp2 to Kp1.[1]

(2) Protein complex dissociation limits the rate of reductant-dependent but not reductant-independent ATP hydrolysis. This suggests that the electron transfer site is buried at the interface of Kp2 with Kp1 but that the ATP hydrolysis sites are on the surface, possibly bridging the two proteins.

[1] R.N.F. Thorneley, G. Ashby, J.V. Howarth, N.C. Millar and H. Gutfreund (1989) Biochem. J. 264, 657-661.

L-08

Mo-independent nitrogenases of Azotobacter.

Robert R. Eady. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

It is now clear that in Azotobacter species there are three genetically distinct nitrogenase systems, one based on Mo - similar to those of many other nitrogen-fixing organisms, another on V, and a third which appears to contain only Fe. Which of these systems is expressed is dependent on the availability of Mo and V in the growth medium. In the presence of Mo the Mo-independent 'alternative' nitrogenases are not synthesised, a regulatory phenomenon which delayed their discovery by many years.

V-nitrogenases have been purified from Azotobacter chroococcum and A. vinelandii and the third nitrogenase from A. vinelandii. These nitrogenases are comprised of a specific Fe protein and a VFe protein in V-nitrogenase or in the third nitrogenase, a protein which contains Fe and only low levels of Mo or V. As for Mo-nitrogenase activity requires both proteins MgATP and a low potential reductant.

The structural genes of V-nitrogenase and the third nitrogenase have been cloned and sequenced. The derived amino acid sequences show homology to each other and to those of Mo-nitrogenases. In particular, amino acid residues thought to be involved in ligation of redox centres are conserved.

Spectroscopic studies of the VFe protein are consistent with the presence of redox centres analogous to the 'P' clusters of MoFe proteins and a polynuclear cluster containing V, S and Fe. The immediate chemical environment of V in VFe proteins is very similar to Mo in MoFe proteins, and extraction of VFe protein with NMF extracts a vanadium- and iron-containing cofactor centre (FeVaco). Extracted FeVaco has very similar chemical, biological and spectroscopic properties to FeMoco.

Pre-steady-state kinetics of electron transfer in V-nitrogenase are very similar to those of Mo-nitrogenase. A characteristic of both alternative nitrogenases is the reduction of C_2H_2 to form some C_2H_6 in addition to C_2H_4, a reaction which occurs in vivo and provides a test for the functioning of those nitrogenase systems.

Recent advances in our understanding of these systems will be presented and reviewed.

L-09

PROGRESS ON THE STRUCTURE OF THE NITROGENASE IRON PROTEIN FROM *AZOTOBACTER VINELANDII*

Millie M. Georgiadis, Division of Chemistry and Chemical Engineering, California Institute of Technology, 147-75CH , Pasadena, CA 91125

Our recent crystallographic efforts have concentrated on crystals of iron (Fe-) protein from *Azotobacter vinelandii*, space group $P2_1$ with a=57.8Å, b=92.9Å, c=63.6Å, β=100°, containing one dimer in the asymmetric unit. We have used the following to provide phasing information (1) native and anomalous scattering measurements from the iron-sulfur cluster, (2) isomorphous heavy atom derivative data, (3) phase refinement by non-crystallographic symmetry averaging, and (4) atomic refinement of an initial structural model to provide additional phase information. In our recent 3.0Å electron density maps, extended regions of connected chain and secondary structure are evident. A general view of the secondary structure arrangement of the Fe-protein is shown in Figure 1. A detailed fitting of the of amino acids to the electron density has not yet been completed. Our current model of the Fe-protein includes about 70% of the polypeptide main chain atoms. Several general observations about the Fe-protein structure may be noted at this stage: (a) The Fe-protein dimer has a two-fold symmetry axis that relates the two subunits. (b) The cluster is on the surface of the molecule and provides the principal connection between the subunits. Each subunit contains a single domain. A cleft straddling the two-fold axis separates the subunits. (c) The folding pattern of an Fe-protein monomer is of the α/β type, as is generally observed in nucleotide binding proteins (i.e. the Rossmann fold). (d) The ATP binding site is most probably located in the cleft between the two subunits. (e) An α helix ,which we believe contains residues 98 to ≈115 based on the position of cluster ligand 97, is exposed at the top of the molecule and may represent a major site of interaction for binding to the MoFe-protein. This is consistent with crosslinking studies done by Howard *et al.*

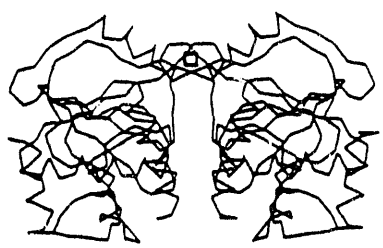


Figure 1. Chain-trace illustrating general features of the Fe-protein dimer structure.

L-12

Nitrogen cycling and human intervention.
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MT-TNO (Netherlands Organization for Applied Scientific Research
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Present-day anthropogenic fluxes of elements like nitrogen, carbon, phosphorous and sulphur are comparable to those of natural origin.
The timescale of the underlying anthropogenic processes is short compared to the natural residence time and the carrying capacity of these elements. This means that environmental compartments tend to become enriched with the elements. In the case of nitrogen we fix too much. Enrichment with nitrogen compounds shows up in all environmental compartments at different geographical and time scales. This has serious potential consequences e.g. interference with the ozone layer, greenhouse effect, eutrophication of fresh water and saline estuaries, soil and groundwater contamination, interaction with biomass and last but not least human health. Elemental cycles interfere with one another. This is especially obvious in higher industrialized and densely populated countries with intensive agro-industrial activities such as Western European countries. These countries are in fact environmental laboratories that are forced to take action to alleviate the immediate and long term consequences of detrimental environmental effects. Quite often this is difficult because of different political systems, legislative and regulatory approaches, different public awareness and different economic conditions in neighbouring countries (e.g. Western and Eastern European nations).
In general long term environmental research and action plans are called for, focusing on how to close elemental cycles, how to conserve energy, how to reuse rather than to dispose and how to emphasize product quality over product quantity.
Biotechnology seems to offer promising contributions in this regard but should be given a healthy dose of scepsis as well.

L-13

Structure, Function and Evolution of Nitrogen Fixing Root Nodules: Confessions of Ignorance.
J. I. Sprent, Department of Biological Sciences. University of Dundee. Dundee. DD1 4HN. Scotland, U.K.

Legumes almost certainly evolved in the tropics, but nitrogen fixation by legume nodules was first studied in Europe, on agricultural species of more recent origin. This led to a set of rules being drawn up, beginning at a root hair infection and culminating with the development of nitrogen-fixing bacteroids enclosed only by a membrane of (largely) host origin. Some variation in rules may be allowed (e.g. choice of *Rhizobium* or *Bradyrhizobium*, usually coupled with export of amides or ureides) but breaking the main rules leads to disqualification – nitrogen fixation does not occur.

Within this framework, great strides have been made in understanding how the symbionts interact at the molecular and short distance (< 100 nm) levels. However, if we move to the whole nodule (or even worse, plant) level major questions remain to be answered including
(a) How is oxygen transported from soil air to N-fixing cells? Is there really a variable resistance to O_2 diffusion and if so how does it operate?
(b) How do nodule water and solute transport systems operate? Is there significant direct exchange with the soil solution?
(c) Is the system really mutualistic? What do the bacteria get out of it? Plants appear to be the dominant partners – some produce nodules without rhizobia – controlling e.g. *Hup* expression. Soon we shall be able to throw the correct switches to produce nodules – getting bacteria inside their cells may be more difficult.

Returning to the tropics, life gets even more interesting. 'Oddities' such as wound infections of *Arachis*, leading to nodules which never contain infection threads, abound. Some such nodules form on stems, as long as they have the potential to form adventitious roots. New genera of rhizobia are being found – *Azorhizobium*, *Photorhizobium* – with novel properties, especially ability to fix N and assimilate products of fixation *ex planta*. Infection pathways between intact epidermal cells (many legumes lack root hairs), nodules where rhizobia are never released from infection threads, and large (7 cm × 7cm) woody nodules have been reported.

Thus we now have many variants on infection systems, nodule morphogenesis and bacterial inhabitants in legume nodules. Indeed, nodules may have only one unique character, namely a stem-like anatomy. An ancestral nodule, formed on stems of primitive legumes growing in flooded soils, following casual infection by a photosynthetic N-fixing ancestor of modern rhizobia is proposed.

In contrast, actinorhizal plants and *Parasponia* have a consistently root-like anatomy. They show considerable variation in their structure, particularly in how they control oxygen supply (anatomical modifications, ± haemoglobin, ± *Frankia* vesicles with a variably thick wall). Although once thought to be largely temperate, they are now known to occur much more widely. Have these variations in nodule composition evolved in response to different environmental constraints? Do they have parallels in legume nodules?

Reports of 'artificial' nodulation of higher plants appear sporadically. Why do they not fix significant quantities of nitrogen? Can we really re-evolve a functional nodule on a legume, other dicots or even monocots? In theory it only needs a few genes, but will it work?

L-14

SURFACE INTERACTIONS BETWEEN RHIZOBIA AND LEGUME ROOT HAIRS

JAN W. KIJNE
Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands

In many rhizobia-legume associations, emerging root hairs are primary target cells for infection. Tight curling of root hairs (Hac) and initiation of a tip-growing infection thread (Inf) require a physical interaction between the bacteria and the root hair surface, in contrast to other nodulation-related phenomena like root hair deformation (Had), root hair branching (Hab), development of thick short roots (Tsr), induction of a nodule primordium (Noi) and induction of infection-related nodulin gene-expression. A computer simulation model (Van Batenburg et al., 1986) predicted that tight root hair curling results from induction/redirection of tip-growth by attached rhizobia and, consequently, that the same process underlies the Hac- and Inf-phenotypes.

Attachment of rhizobia to legume root hair tips is a two-step process. In step 1, single bacteria attach to the root hair surface. This process is probably mediated by rhicadhesin, a 14 kDa Ca^{2+}-binding protein that can be isolated from the surface of Rhizobiaceae-cells, including Agrobacterium. In step 2, rhizobia aggregate at the root hair tip on already attached cells, a process in which secreted plant lectin and rhizobial cellulose fibrils may be involved. Cellulose-mediated aggregation is not required for successful nodulation. A contribution of plant lectin to attachment-step 1 depends on its active presence on the outer surface of the root hair cell wall.

Attachment per se is not sufficient for induction of Hac and Inf, and rhizobial signal molecules and surface polysaccharides are additionally required. It has been suggested that by attachment of rhizobia these signal molecules are targeted to receptive sites of the plant cells. If produced by unattached bacteria, the signals would only induce Had and Hab.

Plant lectin is probably involved in Inf. The pattern of pea lectin localization corresponds perfectly with the pattern of pea root susceptibility to infection by R.leguminosarum biovar viciae (Rl viciae). Furthermore, Rl viciae infects and, occasionally, nodulates white clover (hairy) roots after introduction of a pea lectin gene into these roots (Díaz et al., 1989). Lectin molecules may recognize rhizobial signal molecules and/or surface polysaccharides involved in Hac and Inf.

Lectin presence on the pea root surface and susceptibility to rhizobial infection both are developmentally regulated. Lectin first appears on epidermal cells differentiating into trichoblasts, and trichoblasts with just emerging root hairs constitute the majority of lectin-positive cells. Lectin and susceptibility patterns are increasingly xylem-determined and range from random orientation at the stage of emerging root hairs to xylem-determined positioning of lectin-positive root hairs and nodule formation. Gradients of plant growth factors may determine pattern development. One of these factors is a soluble low-Mw component that can be isolated from the stele of the root.

- Díaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg BJJ, Kijne JW (1989). Nature 338, 579-581.
- Van Batenburg FHD, Jonker R, Kijne JW (1986). Physiol. Plant 66, 476-480.

Hac + Inf modeled on the same mechanism.
lectin peters out in the root - only trichoplasts and young RHs
Spot inoculation shows region of NO hairs gives IFs.
Lectin already localized in right cells prior to RH development.
Not known if free lectin is the same as bound lectin

L-15

RHIZOBIUM MELILOTI EXTRACELLULAR NOD SIGNALS

Philippe Roche[1], Patrice Lerouge[1], Catherine Faucher[2], Jean-Claude Promé[1], Georges Truchet[2] and Jean Dénarié[2]
[1] Centre de Recherches de Biochimie et de Génétique Cellulaires, CNRS-UPS, 118 route de Narbonne, 31062 Toulouse Cedex,
[2] Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex, France

The interaction between fast-growing *Rhizobium* species and their leguminous hosts is highly specific. For example, *R.meliloti* strains nodulate *Medicago*, *Melilotus* and *Trigonella* species while *R.leguminosarum* bv. *viciae* strains form nodules on *Pisum* and *Vicia* species. *R.meliloti* genes required for *Medicago sativa* infection and nodulation can be classified as follows: (i) The *nodABC* common genes which are structurally and functionally conserved in all *Rhizobium* species, (ii) the regulatory *nodD* genes and (iii) the *nodFEG*, *nodH* and *nodPQ* host-range genes.

The use of biological assays (1,2,3) has revealed that the sterile supernatants of *R.meliloti* cultures, after induction of the *nod* genes, contain factors which provoke plant reactions (2,3). The common *nodABC* and the specific *nodH* and *nodQ* genes are involved in the production of extracellular symbiotic Nod factors (2, 3). The specificity of the supernatants in bioassays correlates with the specificity of infection and nodulation of the bacteria (2, 3).

We have constructed *R.meliloti* derivatives which carry either the wild-type *nod* region or mutations in *nodH* or *nodQ* genes and which overproduce the Nod factors (4). These Nod factors were purified by gel permeation, ion exchange and C_{18} reverse-phase high performance liquid chromatography. Their structure was determined from mass spectrometry, nuclear magnetic resonance and chemical modification studies (4). Purified Nod factors were active in specific bioassays in the nanomolar range.

To interpret our biological and biochemical data we propose the following model:

1. The common *nodABC* genes code for proteins which modify a cellular component synthesized independently from the *nod* genes, into a common Nod factor;
2. Host specific genes, namely *nodH* and *nodQ* in *R.meliloti*, code for proteins which allow the modification of a common Nod factor into specific Nod signals; inactivation of individual host-range genes results in the accumulation of intermediate Nod factors which have a modified or reduced host-specificity (resulting in a shift or extension of the host-range);
3. *R.meliloti nodABC*, *nodH* and *nodQ* genes determine alfalfa infection via these extracellular factors.

1. Zaat S.A.J, van Brussel A.A.N, Tak T., Pees E.E. and B.J.J Lugtenberg (1987) J. Bacteriol. 169:3388-3391.
2. Faucher C., Maillet F., Vasse J., Rosenberg C., van Brussel A. A. N., Truchet G. and J. Dénarié (1988) J. Bacteriol. 170:5489-5499.
3. Faucher C., Camut S., Dénarié J. and G. Truchet (1989) Molec. Plant-Microbe Interact. 2:291-300.
4. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C. and J. Dénarié (1990) Nature, in press.

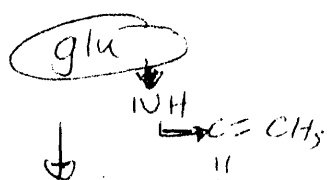
NodRm1 ($+SO_4^{=}$) – induces Sed + Nod.
NodRm2 ($-SO_4$)

L-16

Isolation, Characterization and Structural Elucidation of a "nod signal" Excreted by Rhizobium trifolii ANU843 Which Induces Root Hair Branching and Nodule-Like Primordia in Axenic White Clover Seedlings. Rawle I. Hollingsworth[1], Saleela Philip-Hollingsworth[1], and Frank B. Dazzo[2]. Departments of Biochemistry and Chemistry[1], and Department of Microbiology & Public Health[2], Michigan State University, East Lansing, MI 48824.

We have been studying several extracellular metabolites from Rhizobium trifolii ANU843 which require flavone nod-gene inducers for production and/or export. One of these metabolites was isolated by a combination of C-18 reverse phase chromatography and HPLC-gel permeation chromatography. Purity was established on TLC using both amino-propyl and cyano-bonded silica layers. Similar TLC analyses indicated its absence in crude culture filtrates from R. trifolii ANU843 grown without flavone and also from a nodD::Tn5 mutant derivative (R. trifolii ANU851 from B. Rolfe, ANU). We have established its structure by FTIR, proton NMR, FAB/MS, and chemical methods. We have detected several biological activities of the purified molecule on axenic roots of white clover seedlings, including the following morphological responses: (i) enhanced differentiation of root epidermal cells into root hairs; (ii) branching of developing root hairs; (iii) swelling of tips of matured root hairs accompanied by new root hair elongation at swollen tips; and (iv) development of foci of cortical cell divisions resulting in nodule-like meristems. Thus, it is a "nod signal" requiring inducer flavones and nod gene(s) for production and/or export and contributes to the Hap (hair production), Hab (hair branching), and Noi (nodule initiating) symbiotic phenotypes of wild type R. trifolii.

Independent of flavone:
BF1 – root hair proliferation (wiederum wieder weiche Wissenschaft)
BF2 – root hair curvature
Flavone dependent:
BF3 – Hair defective Noi
BF4 – enhances BF-3, Noi
BF5 = branchy, swelly, weak / Noi
– N-acetyl glutamic acid
0.6 foci/plant // 0.5 mg/4 Liter
– also stimulates wt induced foci

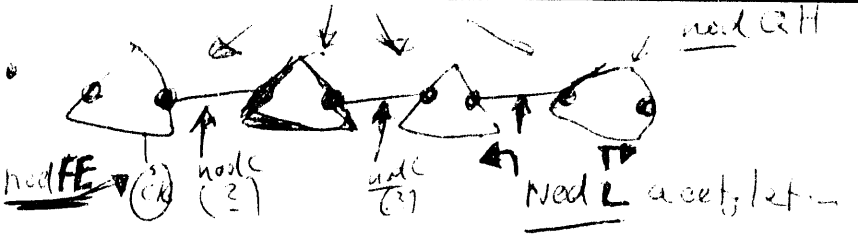


BF6 – thickening of cortex
– optimal = 10^{-11} g/seedling/8 µl
$\cong 10^{-5}$ M

nodM – glucosamine synthase

Symbiotic vs
non-symbiotic genes

L-17



Exported proteins encoded by nod genes from Rhizobium leguminosarum biovar viciae. J.A. Downie*, A. Economou, W.D.O. Hamilton+ and A.W.B. Johnston, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK and +Agricultural Genetics Company, c/oCambridge Laboratory, AFRC Institute of Plant Science Research, Maris Lane, Cambridge CB2 2JB

In R. leguminosarum bv. viciae, 13 nodulation (nod) genes have been identified; nodD, nodABCIJ, nodFEL, nodMNT and nodO. Two of these genes, nodO and nodT encode proteins that have characteristics of proteins exported by bacteria.

The predicted protein sequence of nodO indicated that it is homologous to a group of bacterial proteins (including haemolysin, leukotoxin and proteases) that are exported into the growth medium of Gram negative bacteria. The NodO protein was found to be present in the growth medium of strains induced for nod gene expression and N-terminal sequencing of the NodO protein indicated that it (like haemolysin) is exported by a mechanism that does not involve N-terminal processing. Since a strain lacking its symbiotic plasmid but carrying the cloned nodO gene exported NodO protein it appears that the genes required for the export of NodO are not on the symbiotic plasmid. Significantly the NodO protein was found to bind Ca^{2+} suggesting a role for Ca^{2+} mediated attachment of NodO protein to plant cells.

Although transposon induced mutations of nodO did not inhibit nodulation, deletions of the nodFELMNTO region blocked nodulation completely. Nodulation by this deletion mutant could be restored by plasmids carrying either of two unrelated regions of DNA, one containing nodO or another containing nodFE(L). Therefore the nodO gene product can functionally substitute for the host-specific nodFE(L) genes. This observation is particularly surprising since nodO encodes as expected protein whereas the nodFE(L) genes appear to be involved in the formation of a low molecular weight signal molecule.

ΔFELO MNT = nod⁻ + EPS⁻ (Nod⁻)
↓
Nod⁺ (cellular complementation)

NodO substitutes for nodFEL
Lhaemolysin related.
= 43K (50Kd in gene)
in decoration
Ultimate match.

nodF = fatty acyl transferase protein

nodE ≅ fabB – fatty acyl transferase
keto synthase

nodL = lacA = the galactoside acetyl transferase.

Acetate labeling.
RT-C18 Acetonitrile H₂O
Lipid extract chromatography

nodT = transport system membrane protein

nodM = glmS = glucosamine phosphate synthetase

L-18

Nutrient exchange across the peribacteroid membrane

David A. Day, Botany Department, Australian National University, Canberra A.C.T., 2601, Australia.

The peribacteroid membrane (PBM) is a plant-derived membrane which surrounds the nitrogen-fixing bacteroid in legume nodules, effectively separating the endophyte from the host cell's cytoplasm. The PBM must obviously play a key role in the regulation of metabolite exchange between bacteroid and plant and has the potential to control the symbiosis. In legume-*Bradyrhizobium* symbioses, a single PBM may enclose many (up to twenty) bacteroids, to form large peribacteroid units (PBUs). We have exploited the large difference in density between PBUs, free bacteroids and plant organelles to isolate and purify PBUs from soybean and siratro nodules in a rapid and simple procedure utilizing Percoll gradients. These large PBUs are ideally suited to transport mesurements by the silicon oil centrifugation method.

Sucrose uptake into PBUs and bacteroids is negligible and this compound can be used to estimate internal volumes of units and cells. Glucose and fructose uptake by PBUs is very slow and occurs as passive diffusion; this is also true for glucose uptake by bacteroids, but fructose uptake by the endophyte is more rapid and is catalysed by carrier systems.

Uptake of a range of aminoacids by PBUs has been examined, with attention focused on glutamate and proline since they have been implicated in supply of carbon and reducing power to bacteroids. We have obtained no evidence for aminoacid carriers on the PBM although the bacteroids do possess such carriers and can take up glutamate, in particular, rapidly. In general, aminoacid uptake by PBUs is slow and occurs via passive diffusion. Nonetheless, extensive labelling of bacteroid proteins occurs when intact PBUs are incubated for 60 min with ^{35}S-methionine. Very little labelling of PBM or PBS (peribacteroid space) proteins is observed with isolated soybean PBUs.

Malate and succinate are rapidly transported across the PBM via a dicarboxylate carrier which also recognizes oxaloacetate, fumarate and, weakly, malonate. Malate and succinate uptake by PBUs is rapid enough to account for observed nitrogenase activity in isolated bacteroids. Competition experiments with a range of organic acids and inhibitors clearly show that the PBM dicarboxylate carrier is distinct from those described on the tonoplast, plastid, mitochondrial and bacteroid membranes. Malate uptake by PBUs is severely inhibited by uncouplers and poisons of bacteroid respiration.

The PBM possess an ATPase which displays P-type properties and which can catalyse formation of a $\Delta\psi$ across the PBM of intact PBUs. This $\Delta\psi$ is collapsed by uptake of several anions, notably nitrate and choride, and results indicate the presence of an anion channel on the PBM which is distinct from the dicarboxylate transporter. Anion uptake converts $\Delta\psi$ to a ΔpH, although the transition is slow. Malate uptake is also at the expense of the $\Delta\psi$ and is stimulated to some extent by ATP and inhibited slightly by nitrate. ATP has no effect on sugar or aminoacid uptake.

Ammonia transport across the PBM has been investigated using [^{14}C]-methylamine. There is no evidence for carrier-mediated transport across either bacteroid or peribacteroid membranes and ammonia efflux during nitrogen fixation must occur as diffusion driven by a concentration gradient.

L-19

IMMUNOLOGICAL DISSECTION OF THE PLANT-MICROBIAL INTERFACE IN PEA NODULES.

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Rhizobium leguminosarum biovar *viciae* induces cortical cell hypertrophy on *Pisum* and *Vicia* pea roots. It invades the host plant tissue, first as an intercellular endophyte and subsequently as an intracellular symbiont. The object of the present study was to identify macromolecules involved in plant-microbe surface interactions using rat monoclonal antibodies as molecular probes.

A group of extracellular plant glycoproteins has been identified(1). Immunocytochemical studies have been undertaken to investigate the distribution of these matrix glycoproteins in intercellular spaces in developing *Vicia* roots soon after inoculation with *Rhizobium*, and in longitudinal sections of mature root nodules of *Pisum*. One monoclonal antibody recognised a 95 kDa antigen that was present in *Vicia* seedling roots in the lumen of growing infection threads and in the intercellular spaces of the root cortex underlying curled root hairs. In longitudinal sections of pea nodules this same antigen was abundant in the post-meristematic zone which is associated with intercellular penetration of rhizobia and the development of infection threads. A second monoclonal antibody identified another group of extracellular glycoproteins present in intercellular spaces adjacent to a narrow band of uninfected cells in the inner nodule cortex (the boundary layer). A third monoclonal antibody identified an epitope that was common to both the above classes of glycoprotein. Induction of the synthesis of matrix glycoproteins is an early and probably an important host-plant response to *Rhizobium* infection.

When individual bacteria are released from the infection droplet into the plant cell cytoplasm, they develop into endosymbiotic nitrogen-fixing bacteroids. These bacteroids are individually enclosed by a plant-derived peribacteroid membrane, which is antigenically similar to plant cell plasma membrane(2). Four classes of antigenic macromolecule will be described. Golgi-derived glycoproteins and glycolipids form a glycocalyx, which projects into the lumen of the peribacteroid space, and may become associated with cell wall components of the endosymbiotic bacteroids.

The bacteroid surface carries a form of bacterial glycolipid (lipopolysaccharide) which is antigenically modified compared to free-living bacteria(3). Low oxygen or low pH culture conditions have been shown to induce the lipopolysaccharide antigens that are expressed in the pea nodule(4,5). A combined genetical and immunochemical analysis of *Rhizobium* lipopoly-saccharide has been undertaken. Certain key epitopes have been identified which must be present for the development of the nitrogen-fixing symbiosis.

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pH equilibrium ; lytic vesicle (acid)

L-20

Oxygen diffusion in the legume root nodule. J.F.Witty* and F.R.Minchin. Department of Environmental Biology. Welsh Plant Breeding Station. Aberystwyth, Dyfed SY23 3EB. UK.

Oxygen diffusion into the legume nodules is controlled by a barrier which is rapidly adjusted to balance inward flux with respiratory consumption. Dark field microscopy, electron microscopy and measurements of H_2 and O_2 profiles through nodules together with mathematical simulations suggest that the major route of O_2 flux is via gas filled pathways which are greatly reduced or absent in the inner cortex. On the inner side this zone, surrounding the core of infected cells, there exists a well developed radial distribution system which connects with a matrix of spaces permeating the infected region.

Indirect evidence for variability of the barrier, based upon changes in CO_2 production in response to acetylene or changes in external O_2 concentrations has been criticised because rates of O_2 consumption have been inferred from CO_2 production. However simultaneous measurements of uptake and production of these gases gave RQ values close to unity. Direct evidence for rapid changes in resistance is provided by transient responses of O_2 selective micro-electrodes to increased gas phase O_2 concentration. Similar transient increases have been observed in the oxidation state of leghaemoglobin and in the rate of CO_2 evolution.

Hydrogen specific micro-electrodes have been used to examine H_2 concentrations and gradients within the nodule and measure diffusion resistance. The latter can be calculated from direct measurements of H_2 flux and external and internal H_2 concentration or, where the volume of internal air spaces is known, from the rate of decay in internal H_2 concentration following the removal of H_2 from the gas phase. This method has been used to measure changes in diffusion resistance of soyabean nodules in response to nitrate and detopping. In addition to an increase in resistance electrodes showed a change in the 2 gradients and concentration within nodule.

Changes in diffusion resistance may be accomplished by alterations in the water filled pathlength through and on either side of a regular layer of thin walled cells in the inner cortex, possibly mediated by the effect of O_2 on osmotic potentials. The presence of glycoprotein occlusions in the intercellular spaces in this region are also likely to be involved in the structure and operation of the diffusion barrier.

L-21

Metabolism of C_4-dicarboxylates and amino acids in soybean nodule bacteroids, and their role to energy supply to nitrogenase.

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Relationships between amino acids and C_4-dicarboxylates metabolism

Bacteroids possess active uptake system of C_4-dicarboxylates, glutamate and aspartate. The compounds significantly stimulated C_2H_2 reduction as well as respiration of bacteroids isolated anaerobically from the nodules. Tracer experiment strongly suggested that the compounds were metabolized through complete citric acid cycle in the bacteroids.

Regulation of C_4-dicarboxylate oxidation by oxygen supply to bacteroids Generation of $^{14}CO_2$ from ^{14}C-organic acids depends on oxygen supply to bacteroids, and was stalled by adding >2 mM succinate. Presence of LbO_2 in the incubation system recovered the CO_2 output.

Malic enzyme as a key enzyme for citric acid operation in bacteroids

Rate of acetyl-CoA supply to citric acid cycle was suggested to limit bacteroid respiration rate, and malic enzyme showed highest activity among anaplerotic reaction enzymes. Cultured B.japonicum contained one NADP-malic enzyme, and nodule bacteroids induced another NADP-malic enzyme. These malic enzymes were inhibited strongly by ADP, ATP, succinate and malonate, suggesting the possibility of regulation by those metabolic intermediates.

Relationships between succinate oxidation and energy status in bacteroids Nucleotide analysis in bacteroids and nodules showed concentration of ADP +/& ATP in the tissue was high enough to inhibit ca. 50% NADP-malic enzyme activity. When the bacteroids were incubated with succinate the energy charge and ATP/ADP ratios were increased as the oxygen concentration in the incubation system changed from 1% to 5, 10 and 20%.

L-22

A genetic approach to analyze the critical role of oxygen in bacteroid metabolism. H. Hennecke*, M. Bott and H.M. Fischer. Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstr. 7, CH-8092 Zürich, Switzerland.

There are two essential processes in endosymbiotic bacteroids for which the ambient oxygen concentration is critical: respiration and N_2 fixation. The apparently paradoxical requirements of respiration for oxygen supply and of nitrogenase for oxygen exclusion are met (i) by the induction of a respiratory chain with a high-affinity terminal oxidase that functions at nanomolar free oxygen, and (ii) by a sophisticated genetic redox control system that allows nitrogenase gene expression only at these low O_2 tensions.
Our laboratory is engaged with studies aimed at elucidating the molecular genetic basis of both processes (i) and (ii) in the soybean root nodule symbiont, *Bradyrhizobium japonicum*. We have obtained a set of Tn*5* mutants defective in various components of the respiratory chain(s). Their analysis has shown that *B.japonicum* contains at least three respiratory chains one of which is similar to the one in mitochondria: $2[H] \rightarrow Q \rightarrow FeS/bc_1 \rightarrow c \rightarrow aa_3 \rightarrow O_2$. The fact that mutations in genes for the cytochrome bc_1 complex led to a Fix^- phenotype whereas cyt *c* and aa_3 mutants were Fix^+ suggested an involvement of bc_1 in a putative, bacteroid-specific branch of the electron transport pathway. With regard to *nif* and *fix* gene control, the NifA protein has been recognized as being the crucial redox-responsive gene regulator. Our progress on the analysis of *nifA* gene expression and NifA function will be reviewed (see also the abstracts of D. Anthamatten and H.M. Fischer).

fbcFH operon (F) (H)

Rieske FeS protein

Cyt b — Cyt c_4

Cyt M = 20K – cyt C – membrane bound.

Con A = aa_3 (56K)

– In vitro assay for nifA protein activation + O_2 inactivation.

L-23

Molecular Analysis of Terminal Oxidases in Electron Transport Pathways of Bradyrhizobium japonicum and Azotobacter vinelandii.

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Both B. japonicum and A. vinelandii contain branched electron transport pathways, utilizing multiple terminal oxidases. It is thought that the roles the multiple oxidases play in N_2 fixation are to permit both coupled ATP-synthesizing, and uncoupled but O_2-scavenging electron transport (respiratory protection). In this way, the organism may protect nitrogenase from O_2 damage, while synthesizing sufficient ATP for N_2 fixation. Some of the genes encoding cytochromes involved in O_2 consumption have been cloned; this will allow a molecular analysis of their regulation and help in assigning specific O_2 scavenging or ATP-synthesizing roles to the specific oxidases.

Free-living B.japonicum express cytochromes aa_3 and cytochrome o as the major terminal oxidases, but the cells isolated from root nodules contain a complement of carbon monoxide reactive heme proteins different from those in free-living cells. These include cytochromes P-450,P-420, c-552 and c-554 (see Appleby manuscripts). In addition, an atebrine-sensitive cyanide-reactive flavoprotein with a relatively high redox potential is apparently responsible for a large portion of the cyt c oxidase activity of bacteroid membranes. Evidence that a cyt c oxidase purified from bacteroid membranes is a glycoprotein is presented at this congress (Keefe and Maier, see Abstract). The a-type cytochromes are commonly associated with bacterial cultures incubated in high O_2 environments and are usually not expressed in Rhizobium isolated from nodules. Previously we cloned a B. japonicum cytochrome aa_3 gene; the plasmid pCA1 complemented a cyt aa_3 B. japonicum mutant both spectrally and functionally (O'Brian and Maier, Proceed. Natl. Acad. Sci.,1987). Analysis of the nucleotide sequence and the derived amino acid sequences revealed extensive areas of homology with mitochondrial and prokaryotic cyt aa_3 sequences. The sequenced portion is 74% homologous with yeast cyt aa_3 subunit 1.

The respiratory chain of Azotobacter vinelandii is also branched at the terminal oxidase steps, involving either cytochrome d or o as terminal oxidase. We have cloned and sequenced the structural gene for cyd of A. vinelandii. Two open reading frames encoding proteins with extensive areas of homology to subunit I and subunit II of the E. coli cyt d complex are present (Moshiri, et. al., see Abstract). The sequence of the region upstream of the initiation codon for subunit I revealed a ribosome-binding site and a putative promoter region which is similar, but not identical to the canonical nif promoters of A. vinelandii.

L-24

Measurement of N_2 fixation by ^{15}N natural abundance, in the management of legume crops: roles and precautions. F.J. Bergersen, CSIRO Division of Plant Industry, Canberra, A.C.T. 2601, Australia.

Goals for research include improved utilization of N_2 fixing components of agricultural systems and in the conservation of forests or fragile ecosystems. Evaluation of success in attaining these goals will be aided by more accurate quantitative assessment of levels of N_2 fixation actually achieved in the field. The proportion (P) of plant nitrogen arising from N_2 fixation may be estimated from small differences in the natural abundances of ^{15}N (measured as $\delta^{15}N$ in parts per 10^3 relative to air N_2; o/oo) between plant-available combined N in the soil and plant N fixed from N_2 (review by Shearer & Kohl 1986). Thus:-

$$P(\%) = 100(x-y)/(x-c) \qquad (i)$$

where x,y and c are respectively the $\delta^{15}N$ of the plant-available soil N, of the total N of N_2-fixing plants growing in that soil and of the total N of plants which are completely dependent on N_2 fixation for growth.

Measurements of $\delta^{15}N$ have been made in the total N of plants and in extractable mineral N of soil, with adequate precision in Australia and S.E. Asia. For example, in most experiments the mean $\delta^{15}N$ of plants totally-dependent on soil N for growth was in the range 4 to 17 o/oo and the s.e. of the means 0.1 to 0.5 o/oo. Precision of measurement depended entirely on strict adherence to methods which minimised or eliminated errors due to isotopic discriminations occurring at sensitive stages in the analysis. Some of these possible discriminations will be described.

The natural abundance method has been used to measure P in field crops of soybean, ricebean, green and black grams, peanut, lupin, desmodium, subteranean clover and lucerne, in collaboration with other workers who will be identified in the printed paper. Amounts of N_2 fixed per ha ranged from 13 to 260 kg N in growth periods of 25 to 193 days. Representative data will be presented illustrating the use of the method in field experiments investigating the effects of cropping sequences and rotations, intercropping legumes and non-legumes, legume variety, inoculation level, water relations, season, cutting height in forage legumes, and pasture composition. Examples will also be given of the use of the method in apportioning the contributions of fixed N_2 to seed N and quality of soybeans and to the contents of N in the components of the crop at harvest in relation to the N-balance.

Where the natural abundance method has been compared with the ^{15}N enrichment method (reviewed by Chalk 1985) we have obtained similar estimates of N_2 fixation but a few discrepancies occurred, particularly at low values of P. Similar estimates of P were also obtained when the $\delta^{15}N$ of plant-available soil N (x in the expression (i) above) was obtained from analysis of un-nodulated plants, non-nodulating genotyes or the extractable mineral N of the top 10 cm of the soil. Again there were some discrepancies at low levels of P. Recently, the natural ^{15}N abundance method has been used in the field to calibrate the xylem ureide method for estimating P.

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L-25

Map position of sym genes involved in nodulation and nitrogen fixation in Pisum sativum. N.F. Weeden, B.E. Kneen and T.A. LaRue* Department of Horticultural Sciences, Cornell University and Boyce Thompson Institute, Ithaca, NY 14853, USA.

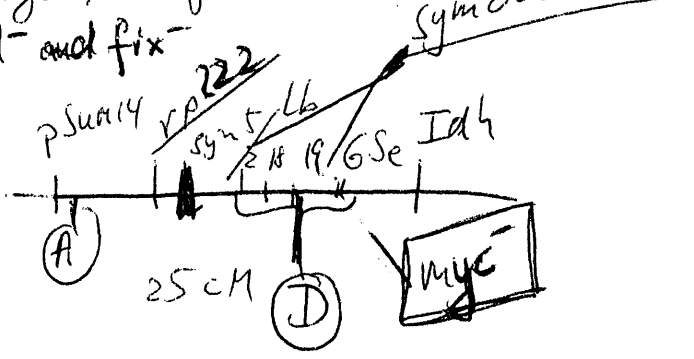
At least 17 genes affecting nodule formation or encoding nodule-specific proteins have been located on the chromosomal linkage map of the garden pea, Pisum sativum L. Most of these genes were identified by treating the cultivar 'Sparkle' with mutagens and screening the M2 progeny for abnormal nodulation; however, the leghemoglobin genes and two nodule-specific glutamine synthetase loci also have been mapped. Nearly half of the nodulation-impaired mutants mapped near the major cluster of leghemoglobin genes on chromosome 1. The other mutants were located on five of the six remaining chromosomes. Of these latter mutants sym-13, which produces a non-fixing nodule, mapped near one of the loci encoding nodule-specific glutamine synthetase. No other indication of clustering of loci involved in nodule formation or metabolism was found. Thus, the region adjacent to the major leghemoglobin cluster on chromosome 1 appears to be either unusually labile to mutation or particularly important for the nodulation process. None of the clustered induced mutants on chromosome 1 showed any abnormality in leghemoglobin expression when nodules were formed. The phenotypes of these mutants, sym 2, sym 5, sym 18 and sym 19 will be described.

7 chromosomes, tester like, 1 year, lots / greenhouse.

[5 cupsLB on / 1 on r] 21 nod⁻ and fix⁻

GS_{n_1} } — 6, 7
n_2 }

GSCy⁻ 3 (little on ①)
GSp⁻ 1



ES — short last roots
— first 2 short internodes
— all root controlled —

sym5 — ts for root temp. → hot spot → done by reverse of ethylene antagonists

Δ 66Kd in vivo / in vitro
pI 5.9

AVE 10-6M
$Co^{+}(NO_3)_2$ 10-4M
$AgS_2O_3^{-1}$ 10-6M

L-26

Host legume control of nodulation and other rhizosphere events through release of flavonoids

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Recent demonstrations show that plant flavonoids are powerful transcriptional activators of nodulation (*nod*) genes in rhizobia. As one step toward further defining *Rhizobium*-legume interactions and understanding how flavonoids influence other microbes in the rhizosphere, we are identifying compounds released naturally from alfalfa (*Medicago sativa* L.) and common bean (*Phaseolus vulgaris* L.). Using spectroscopic methods (UV/visible absorbance, NMR, MS), HPLC comparisons with authentic standards, and assays with *nod-lacZ* fusions in *Rhizobium meliloti*, we have identified flavonoids that are released by 'Moapa 69' alfalfa during the first 72 h of germination (Maxwell *et al.*, Plant Physiol. 91:842-847, 1989; Hartwig *et al.*, Plant Physiol. 92:116-122, 1990). The major *nod*-gene-inducing flavonoids released from seeds are luteolin and chrysoeriol (3'-methoxyluteolin), which is three times more active than luteolin as a NodD1 activator. No luteolin was found in root exudates, but 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2'-methoxychalcone were identified as *nod*-gene inducers. The methoxychalcone is 10-fold more active than luteolin as a NodD1 activator and also shows strong NodD2 activation (Hartwig *et al.*, J. Bacteriol. In press). Mixtures of these compounds show both promotive and inhibitory effects in *nod*-gene-induction assays (Hartwig *et al.* Plant Physiol. 91:1138-1142, 1989). Luteolin and the methoxychalcone, which probably occur together in the soil at the top of the primary root, show positive synergistic effects on *nod-lacZ* transcription.

Several phenomena related to release of *nod*-gene-inducing flavonoids offer potential control points for the host plant. Exudation of the inducer molecules from alfalfa roots apparently requires concurrent synthesis (C.A. Maxwell, Ph.D. thesis, 1990), and enzymatic activity present in sterile alfalfa seed exudate can convert luteolin-7-O-glucoside, which is not a *nod*-gene-inducer, into luteolin (U.A. Hartwig, unpublished data). *R. meliloti* also can degrade luteolin-7-O-glucoside to luteolin.

Whether nodulation patterns are related to the flavonoids exuded by legumes remains to be determined. Alfalfa populations differing in root nodule number had no major changes in NodD1 activators, but flavonoid mutants of bean show a more direct relationship between nodulation and *nod*-gene inducers released.

The presence of structurally different flavonoids in seed and root zones of alfalfa seedlings offers the possibility of different effects on biological functions, such as chemotaxis. Preliminary studies show highly significant biological effects of these compounds on microbes present in alfalfa soils, and indicate that knowledge developed from study of the *Rhizobium*-legume symbiosis will assist efforts to understand other plant-microbe interactions.

L-27

H_2 cycling in N_2 fixation: Past, present and future outlook, D.J. Arp, Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, OR 97331, USA.

As early as 1938, a unique relationship between dihydrogen metabolism and N_2 fixation was recognized with the observation by Wilson and colleagues that H_2 is a specific and competitive inhibitor of N_2 fixation. In 1957, Hoch, Little and Burris observed H_2 evolution by soybean root nodules. In 1968, ROD Dixon demonstrated that H_2 evolution occurred simultaneously with D_2 uptake in pea root nodules which indicated that both an a H_2-oxidizing activity and H_2-producing activity occurred in the root nodule. Eventually, H_2 evolution was shown to be associated with nitrogenase and H_2 oxidation with hydrogenase. However, it was not until the late 1970's, when Dr. Harold Evans directed the attention of his laboratory to this problem, that investigations of the dihydrogen metabolism of legume root nodules entered the log phase of productivity.

Dr. Evans and his colleagues demonstrated the extent of H_2 evolution by nitrogenase in root nodules and revealed that a substantial proportion of the electron flux through nitrogenase was being directed towards H_2 production. They also confirmed that the H_2-oxidizing phenotype was not predominant among the *Rhizobium* and *Bradyrhizobium*. In a series of experiments of ever-increasing sophistication, Evans and his colleagues tested the theory that H_2 oxidation increases the efficiency of N_2 fixation. The experiments demonstrated that plants bearing nodules which expressed hydrogenase generally had higher N content and greater dry mass than those plants bearing nodules which did not express a hydrogenase. Additional contributions were also made in our understanding of the physiological aspects of dihydrogen metabolism, including the pathway of electron flow from dihydrogen to O_2 and the coupling of dihydrogen oxidation to ATP synthesis. One entirely unexpected discovery was that some strains of *Bradyrhizobium* could grow lithoautotrophically with H_2 as the sole source of energy. Molecular investigations of hydrogenase were also carried out. For example, trace element studies revealed that Ni was required for expression of hydrogenase and purification of hydrogenase revealed that hydrogenase contained Ni. Recognizing early on the need to investigate the genetics and, eventually, molecular biology of dihydrogen metabolism, Dr. Evans initiated a major effort in his laboratory in this area. His laboratory was the first to begin mutant selections, establish screening protocols and, ultimately, to clone the genes coding for hydrogenase in a N_2-fixing microorganism. The structural genes for the large and small subunits of hydrogenase have now been sequenced.

Through the contributions of Dr. Evans and his colleagues, and the contributions of other investigators working with symbiotic and free-living aerobes, phototrophs and other N_2-fixing microorganisms, the field of dihydrogen metabolism has achieved the status of a major sub-discipline of biological N_2 fixation research. Investigations of a number of aspects of the dihydrogen metabolism of N_2-fixing microorganisms continue in several laboratories throughout the world.

693-4595

L-28

Ecology of rhizobia, Peter J. Bottomley, Departments of Microbiology and Soil Science, Oregon State University, Corvallis, OR 97331-3804 USA

There is no evidence that inoculation with superior nitrogen-fixing strains can increase legume productivity in soils already containing effective rhizobia. While a better understanding of rhizobia ecology will not in itself lead to enhanced nitrogen fixation, ecological information will contribute to a more comprehensive understanding of the factors which influence the amounts of nitrogen fixed under field conditions, and to the identification of the factors which govern population size, diversity, activity, and turnover of functional groups of microorganisms present in agricultural soils. Over a period of fifty years numerous studies have shown that soil populations of rhizobia can be incredibly diverse. To date, no deliberate attempts have been made to determine whether the subpopulation structure of rhizobia in soil is regulated by global rules, and if so, do these same principles apply to the population structure of other groups of soil microorganisms. Serotyping and multi-locus allozyme electrophoretic typing methods have revealed the existence of a similar number of different subpopulations of rhizobia in soils. While the former method has a long history, including the use of immunofluorescence for studying autecology of rhizobia, the latter method was introduced recently and has the potential to provide information on the genetic relatedness among subpopulations. Since each method has particular strengths not possessed by the other, there is a need to compare subpopulation structure delineated by the two methods. Information from the author's laboratory will be presented on this subject. Controversy has existed for many years regarding the importance of saprophytic traits on nodulating success under competitive conditions. Despite numerous reports describing physiological differences among isolates of rhizobia under laboratory conditions, there is little evidence for these traits influencing ecological success or failure in natural soils. Nevertheless, data will be presented which show that indigenous subpopulations of R. leguminosarum can exist at significantly different densities in soil, and in different states of substrate responsiveness. Trifolium spp. have played a major role in support of either symbiotic or saprophytic traits being the most critical determinants of the outcome of competitive nodulation between mixtures of R. leguminosarum bv. trifolii. Unfortunately, most of these studies were not carried out in the presence of well characterized soil populations, and involved a limited number of representative species of Trifolium. Data will be presented on our attempts to identify representatives from a soil population of R. leguminosarum bv. trifolii which are competitive only on particular clover hosts, non-competitive regardless of clover host, or which are promiscuously competitive.

- ineffective indigenous strains is a false concept,
- competitive nodulation, saprophytic competence, host preference 1:3 concept,
- need for international reports.

L-29

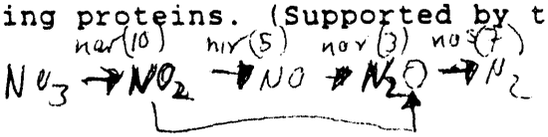
Molecular analysis of the denitrification system of Pseudomonads. W. G. Zumft. Lehrstuhl für Mikrobiologie, Universität Karlsruhe, D-7500 Karlsruhe 1, Federal Republic of Germany.

Bacterial denitrification is an alternative process of energy conservation in the absence of oxygen by facultative anaerobic bacteria. The trait is prevalent among the non-fermentative Pseudomonads. Denitrification can be conceived as the assembly of three losely coupled respiratory processes utilizing as terminal electron acceptors nitrate, nitrite, and nitrous oxide. Four enzymes transforming in sequence nitrate to dinitrogen, i. e. catalyzing the complete denitrification pathway have been isolated and characterized from Pseudomonas stutzeri. The process is of importance for water management (nitrate removal), agronomy (loss of fixed nitrogen), and atmospheric chemistry (NO and N_2O). Knowledge about this process (although discovered before N_2 fixation) is still very fragmentary. An overview of some of the most important features will be given with Pseudomonads as the model organism.

Nitrate reductase is a membrane-bound, heterodimeric, bactopterin-containing iron-sulfur protein (M_r 172 000). It resembles respiratory nitrate reductases from non-denitrifying bacteria. Nitrite reductase is a periplasmic, homodimeric tetraheme protein cytochrome cd_1 (M_r 60 000). From P. aureofaciens a homodimeric Cu protein (M_r 85 000, 2 Cu) with Cu as type I only, has been isolated. Nitric oxide reductase and nitrous oxide reductase are two novel enzymes: a membrane bound, heterodimeric cytochrome bc complex (M_r 55 000) reduces NO to N_2O, whereas a periplasmic, homodimeric multicopper protein (M_r 140 000, 6-8 Cu) reduces N_2O to N_2. The spectroscopic characterization of these enzymes by various techniques with emphasis on N_2O reductase will be summarized.

Random transposon Tn5-induced mutagenesis has been used to generate mutants defective in nitrate respiration (Nar^- phenotype), nitrite respiration (Nir^-), and nitrous oxide respiration (Nos^-). The structural genes for cytochrome cd_1 and N_2O reductase have been identified, isolated, and sequenced. Extended cosmid mapping and Southern hybridizations have revealed that the two genes are located approx. 16 kbp apart on a ca. 55 kbp BamHI fragment. Together with several other genes essential for denitrification there is evidence that denitrification genes of P. stutzeri comprise a gene cluster.

The biosynthesis of metal centers involves within the context of the denitrification process, metal uptake, the synthesis of organic cofactors, protein insertion into or translocation across the cytoplasmic membrane, and the temporal and spatial control of metal and cofactor incorporation in relation to enzyme export. For N_2O reductase three genes have been identified which are required for metal processing or acquisition. The gene products show organizational similarities to bacterial uptake systems for small molecules and comprise a new member of the transport family of ATP/GTP-binding proteins. (Supported by the Deutsche Forschungsgemeinschaft)



L-30

Biochemistry of Nitrification. Alan B. Hooper. Dept. Genetics and Cell Biology, Univ. Minnesota, St. Paul, MN 55108, USA.

Nitrosomonas is an obligately autotrophic and aerobic bacterium which produces energy for growth from the following reactions: $NH_3 + O_2 + 2e^- \rightarrow NH_2OH$ (ammonia monoxygenase, "AMO"); $NH_2OH + H_2O \rightarrow 4\ e^- + 4H^+ + HNO_2$ (hydroxylamine oxidoreductase, "HAO") and $1/2\ O_2 + 2e^- + 2H^+ \rightarrow H_2O$ (terminal oxidase).

At low concentrations of O_2, cells produce a significant amount of NO and N_2O in place of HNO_2. Cells have nitrite reductase activity. Further, HAO, under some conditions of incubation, will produce NO and N_2O directly from NH_2OH. Thus, in theory, N_2O could have been produced in cells by oxidative and/or reductive reaction(s). However, we do not find ^{18}O in N_2O produced by cells in the presence of $^{18}O_2$. Thus *in vivo*, ^{18}O introduced by AMO appears to have been removed by the nitrite/water oxygen exchange reaction known to be catalyzed by cells. The observation suggests that HNO_2 is an obligatory intermediate in the production of N_2O from NH_3 and that all N_2O is produced by reduction.

The electron transfer sequence from soluble, periplasmic HAO to the membrane AMO and cytochrome *aa*3 includes the tetraheme cytochrome *c*-554 and monoheme cytochrome *c*-552. We now find that cytochrome *c*-554, previously thought to be soluble and periplasmic, is an extrinsic membrane protein. It can be eluted at elevated salt concentration from a high-affinity membrane binding site. We have recently measured values of E_o of the 4 hemes of cytochrome *c*-554 (two at +47 mV, one at -147 mV and one at -276 mV). They are in keeping with a possible 2 electron transfer.

By determining the amino acid sequence of all trypsin-generated heme-peptides, the 63 kD subunit of HAO is shown to contain 7 *c*-hemes. One *c*-heme and the chromophore "P460" appear to be involved in the active site. The other hemes are involved in electron transfer or redox poising of the enzyme.

A nitrite reductase from several genera of heterotrophic bacteria catalyzes the reaction; $HNO_2 + 6e^- + 4H^+ \rightarrow NH_3 + 2H_2O$. Interestingly, each subunit (M_r 56 - 66 kD), contains 6 *c*-hemes and is similar in amino acid content to HAO. The possible evolutionary relationship between HAO and these nitrite reductases is being ir vestigated.

By reaction of genomic digests with the appropriate polynucleotide probe, we find that the genome of *Nitrosomonas* appears to have multiple copies of genes for HAO. Plasmids were not detected.

We have recently found that AMO *in vivo* will transform dichloromethane, dibromomethane, trichloromethane (chloroform), bromoethane, 1,2-dibromoethane (ethylene dibromide), 1,1,2-trichloroethane,1,1,1-trichloroethane, *cis*-dibromoethylene, trichloroethylene, and1,2,3 trichloropropane but not tetrachloromethane (carbon tetrachloride), tetrachloroethylene (perchloroethylene) or *trans*-dibromoethylene.

Supported by NSF/DMB 87-19338, USDA/CRGO-88-37120-3957. and EPA/CR-816165.

L-31

Nitrogen Fixation in the Methanogenic Archaebacterium *Methanosarcina barkeri* 227.

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In 1984-85, four laboratories, including ours, described nitrogen fixation in methanogenic archaebacteria. This discovery was the first demonstration of diazotrophy outside the eubacterial kingdom. It also indicates that the methanogens can play hitherto unappreciated roles in nitrogen cycles in anaerobic habitats.

We have been examining the physiology and biochemistry of nitrogen fixation in *Methanosarcina barkeri* strain 227. Nitrogen fixation halved the growth rate and yield of strain 227 when the growth substrate was methanol, and had an even greater effect when the substrate was acetate, which provides less energy than methanol. Molybdate (10 nM) stimulated diazotrophic growth and tungstate (100 μM) inhibited it. Diazotrophic cells reduced 0.003 atm acetylene (higher partial pressures inhibited methanogenesis) to ethylene at extremely low rates, 1-2 nmol h^{-1} [mg protein]$^{-1}$. Cell-free extracts reduced acetylene at higher rates (ca. 25-50 nmol h^{-1} [mg protein]$^{-1}$, but still not at rates that would be expected to support diazotrophic growth.

We have achieved a 20-40-fold purification of the nitrogenase components from strain 227 using a Fast Protein Liquid Chromatograph operated inside an anaerobic glovebox. After protamine precipitation, extracts were fractionated with a Mono Q anion exchange column followed by gel filtration with a Superose 12 column. This allowed rapid purification of the nitrogenase components from the many other iron sulfur proteins present in methanogen cell-free extracts. The activity of the nearly pure components, ca. 1000 nmol h^{-1} [mg protein]$^{-1}$, is about 1% of the activity seen from purified eubacterial Mo-nitrogenase components. A partially purified enzyme preparation reduced N_2 to NH_3 at a rate, per electron, 1.7 fold higher than acetylene. The nitrogenase from strain 227 was binary. One component had a molecular weight (MW) near 240 kdal, with subunit MW's near 57 and 62 kdal. The other component had a subunit MW near 31 kdal, and an anomalously high total MW near 120 kdal, as estimated by gel filtration chromatography. The 31 kdal polypeptide reacted with antibodies to *Rhodospirillum rubrum* component 2 in Western blots.

Adding ammonia, at concentrations as low as 10 μM, caused cells to transiently switch-off acetylene reduction. No switch-off was detected with added glutamate or asparagine, and a brief switch-off from glutamine was attributed to contaminating ammonia. Methionine sulfoxamine had no effect on switch-off. No covalent modification of the 31 kdal polypeptide was detected in Western blots from switched-off cells, and extracts from switched-off cells showed similar acetylene reducing activity to those assayed before ammonia addition.

Low specific activity, a preference for N_2 over acetylene, and lability to freeze-thaw are all features which the nitrogenase from strain 227 shares with the second alternative nitrogenase from *Azotobacter vinelandii*, which contains neither Mo nor V. However, molybdate stimulated diazotrophic growth of strain 227 as well as other methanogens. These results, as well as those of Sibold's group, showing 70% amino acid sequence similarity between an expressed *nifH* open reading frame in *Methanococcus thermolithotrophicus* and the *anfH* of *A. vinelandii*, suggest similarity between methanogen and non-Mo non-V eubacterial alternative nitrogenases.

L-34

Field-release of genetically engineered *Rhizobium meliloti* and *Bradyrhizobium japonicum* strains.

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The aim of BioTechnica International's research and development program in nitrogen fixation is to develop *Rhizobium meliloti* and *Bradyrhizobium japonicum* inoculant products with improved nitrogen-fixing and competitive abilities. Gene cassettes that confer improved nitrogen-fixing or competitive ability are constructed using recombinant DNA techniques. These cassettes are then integrated into specific symbiotically silent regions of the genome of highly effective host rhizobial strains. The recombinant strains are extensively tested under greenhouse conditions to evaluate their nitrogen-fixing and competitive abilities. In order to field-test the strains, pre-manufacture notifications (PMNs) are submitted, in voluntary compliance with EPA policy, to the EPA for consideration under the Toxic Substances Control Act (TSCA). The type of information contained in the submissions will be briefly described. The PMN submissions are also considered by USDA APHIS for determination of the plant pest status of the microorganisms and by state regulatory agencies. However the EPA review, which includes a mandatory public comment period of 90 days, is the primary review. Permission to field-test the strains is granted in the form of a Consent Order, a legally binding document that details protocols and reporting responsibilities. In 1989 BioTechnica received Consent Orders permitting field-testing of recombinant *R. meliloti* and *B. japonicum* strains about 120 days after submitting the PMNs. The field tests were carried out in Sun Prairie, Wisconsin, and Baton Rouge, Louisiana, respectively.

The comprehensive microbiological monitoring program conducted in association with the field tests compared, where possible, the behavior of the recombinant strains to that of their wild-type parents. Parameters followed included aerial dispersal of inoculant on the day of planting, vertical and horizontal dispersal of the inoculant from the site of application, persistence in the rhizosphere, and nodule occupancy. In particular, we were interested in determining whether integration of genes into the selected genomic sites affected the strains' ecological or symbiotic properties under field conditions. It was also important to demonstrate that the population dynamics of the recombinant rhizobia could be monitored. Results of these ecological studies are detailed at this meeting in posters and will be described briefly during this talk.

L-36

Regulation of *nif* gene expression in *K. pneumoniae*.

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The *Klebsiella pneumoniae nif* genes are within a class of bacterial genes which employ a form of RNA polymerase containing the *rpoN* (also called *ntrA*) encoded sigma factor for their expression. Under nitrogen limiting microanaerobic or anaerobic growth conditions expression of the *nifLA* operon is activated by the phosphorylated form of the general nitrogen regulatory protein NtrC. The other *nif* operons are activated by the NifA protein. Analysis of *nif* promoter function *in vivo* and *in vitro* has demonstrated that NifA and NtrC bind to upstream sequences of the promoters which they activate. They then catalyse the isomerisation of a closed promoter complex to the open complex form to allow initiation of transcription (1,2). This process requires the formation of a DNA loop between upstream bound activator and the RNA polymerase recognition sequence. Recent results indicate that the affinity of the RNA polymerase binding site is an important feature of the promoter, determining in part the requirement for upstream-bound activator. Two genes closely linked to *rpoN* appear to influence the activity of *rpoN*-dependent promotors (3).

The above features of *nif* promoter function will be elaborated to include results addressing the repressive function of *nifL* (4), DNA topology requirements for activation of the *nifLA* promoter, the role and location of NifA binding sites in *K. pneumoniae nif* promoters and the action of NifA *in vitro*.

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L-37

Genes Required for Oxygen Regulation in Rhizobium meliloti. M. Gilles-Gonzalez, D. Kahn, A. Lois, M. Weinstein, P. Agron, D. R. Helinski, and G. Ditta.* Department of Biology, Center for Molecular Genetics M-034, University of California San Diego, La Jolla, CA 92093

Oxygen availability is a critical factor governing the transcription of 21 nitrogen fixation-related genes (nif or fix genes) in the alfalfa symbiont R. meliloti. These genes are not expressed during normal vegetative growth, but are induced during root nodule development and during microaerobiosis ex planta. The products of the R. meliloti fixL and fixJ genes (FixL and FixJ) mediate global control of the nif/fix regulon by governing expression of two positively required regulatory genes, nifA and fixK. Based on inferences derived from DNA sequence analysis, FixL and FixJ are members of a large family of regulatory proteins referred to as two component regulatory systems, with FixL expected to be a membrane bound protein responding either directly or indirectly to oxygen, and FixJ expected to be a DNA binding transcrtiptional activator of the nifA and fixK promoters.

Experiments are being conducted to examine the interactions between FixL and FixJ in vivo and in vitro, and to study how such interactions lead to regulatory control of target promoters. Greatly facilitating this work is the fact that both positive and negative regulation of nifA transcription by oxygen can be demonstrated in E. coli. It can be inferred from such data that FixL can respond to oxygen while in a heterologous membrane environment, and that FixJ can interact with E. coli RNA polymerase to effect promoter induction.

In order to carry out a detailed structure-function analysis of FixL and FixJ, various mutants have been created. Several aerobically constitutive mutants of fixL are being analyzed, and a fixL mutant precisely deleted for both transmembrane segments was found to have lost positive (but not negative) function in vivo. Various point mutants of fixJ, leading to either defective or FixL-independent, phenotypes have also been identified. Of particular interest are a number of oligonucleotide-directed mutants that have been created within a helix-turn-helix DNA binding motif in the conserved C-terminal domain of FixJ. Of 29 such mutants targeted to 5 specific amino acid residues, 28 could not induce nifA transcription and 24 could not induce fixK transcription. A deletion derivative of fixJ lacking the N-terminal domain was found to be even more effective than the full length protein at promoting nifA transcription, indicating that the C terminus is specifically responsible for transcriptional activation, and that the N-terminus is likely to negatively regulate such activity.

Both FixL and FixJ are phosphoproteins. After overproduction from a high copy number plasmid in E. coli, FixJ and a soluble, N-terminal deletion derivative of FixL (FixL*) could be phosphorylated with γ-^{32}P-ATP in crude extracts. Considerably higher levels of phosphorylation were observed for both proteins using an analogous deletion derivative of a constitutively active FixL mutant (FixLr2), indicating that phosphorylation of FixJ depends on FixL. Phospho-FixJ was found to be sensitive to alkaline conditions, suggesting the presence of an acyl phosphate. FixL* is currently being purified to confirm that it is an autophosphorylating protein kinase with specificity for FixJ as phospho-acceptor.

Lastly, it is possible to define a concensus FixJ binding site based on genetic studies of the nifA promoter and on comparisons with the fixK and (putative) fixL promoters. Experiments are in progress to examine DNA binding by wild type and mutantpromoters to FixJ.

L-38

Mechanism of transcription from *nif* promoters: involvement of IHF. Eduardo Santero
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The nitrogen fixation (*nif*) operons of Gram negative bacteria are transcribed by an alternative form of RNA polymerase holoenzyme which uses σ^{54} (the gene product of *ntrA*, also called *rpoN*) as a sigma factor. Initiation of transcription from diverse promoters recognized by σ^{54}-holoenzyme has a common feature: it requires an activator protein, which binds to the promoter regulatory region about 100 bp upstream of the transcriptional start site. NTRC is the best characterized activator of σ^{54}-holoenzyme. It has been shown both in vitro and in vivo to catalyze the isomerization of closed complexes to open complexes at the *glnA* promoter of enteric bacteria. To do so it contacts σ^{54}-holoenzyme by means of a DNA loop. NIFA, the transcriptional activator protein for *nif* operons, is structurally and functionally related to NTRC. It has been shown in vivo to bind about 100 bp upstream of *K. pneumoniae nif* promoters and, like NTRC, to catalyze the formation of open complexes [E. Morett & M. Buck. J. Mol. Biol.(1989) 210, 65-77].

We recently showed that integration host factor (IHF) bound to the *nifH* promoter-regulatory region between the binding sites for NIFA and σ^{54}-holoenzyme. We report here that IHF stimulates NIFA-mediated activation of transcription from the *K. pneumoniae nifH* promoter in vitro; stimulation is up to 20-fold. IHF is a small heterodimeric protein involved in many different cellular processes. It binds to specific sites on DNA, producing a sharp bend. We show by electron microscopy that IHF bends a DNA fragment containing the *K.pneumoniae nifH* promoter-regulatory region. We propose a model in which the IHF-induced bend brings NIFA and σ^{54}-holoenzyme closer together, facilitating productive interactions between them.

We have studied in vitro activation of transcription from two stronger mutant promoters as well as from the wild-type *nifH* promoter. Our results suggest that one advantage to use of the "weak" wild-type promoter is to achieve high fidelity of activation by the physiological activator NIFA: NTRC cannot activate from the wild-type promoter but can from stronger mutant promoters. Use of IHF in conjunction with the wild-type promoter allows high fidelity without a sacrifice in the efficiency of activation by NIFA. Other potential advantages to the use of IHF in *nif* expression will be discussed.

IHF not only binds to the *K.pneumoniae nifH* promoter region but also binds to the *K.pneumoniae nifJ*, *nifE*, *nifU* and *nifB* promoter regions and to the *nifH* promoter regions of *Azotobacter vinelandii*, *Rhizobium meliloti*, *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum and Thiobacillus ferrooxidans*. In all cases the IHF binding site is located between binding sites for NIFA and σ^{54}-holoenzyme. These data suggest that the involvement of IHF in *K.pneumoniae nifH* expression is not an isolated case; we predict that IHF will stimulate NIFA-mediated activation of transcription from many *nif* promoters. In addition, these results suggest that IHF is not restricted to enteric bacteria, but is a DNA-bending protein with a well- conserved binding specificity that is widely spread among bacteria.

We have recently demonstrated in vitro activity of NIFA that was synthesized in a coupled transcription-translation system. This activity was markedly unstable, which was in contrast to the stability of NTRC synthesized under the same conditions. The nature of the inactivation is not known, but it is not due to gross proteolytic degradation. It is not clear whether this inactivation pertains under physiological conditions, but it may be related to the problem of obtaining active NIFA protein upon overproduction.

L-39

Organization and regulation of nitrogen fixation genes in Rhodobacter capsulatus

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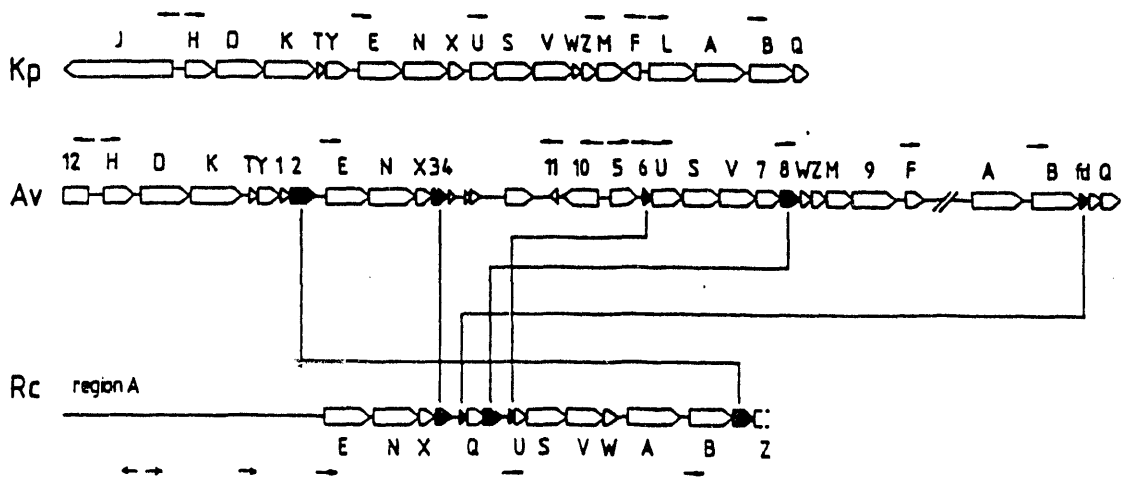
The complete nucleotide sequence of a 13,964 bp fragment of Rhodobacter capsulatus (Rc) nif region A revealed genes homologous to Klebsiella pneumoniae (Kp) and Azotobacter vinelandii (Av) nifE, N, X, Q, U, S, V, W, A, B and Z. In addition to these "common nif genes" five open reading frames (ORFSs) were identified which are not present in the nif gene cluster of K.pneumoniae but have their counterparts in A.vinelandii. The map positions of ORF3 and ORF6 are identical in R.capsulatus and A.vinelandii. In contrast, homologues to ORF2, ORF8 and to an open reading frame encoding a ferredoxin-like protein (fd) are localized at non-corresponding positions.

The DNA sequence analysis of a 7 kb fragment upstream of nifE, which was previously shown to contain genes essential for nitrogen fixation, revealed eight open reading frames. Three of them encode low molecular weight ferredoxin-like proteins. The deduced amino acid sequences of the remaining coding regions, including a 56 kDal protein, which contains two cysteine motifs typical for proteins coordinating 4Fe-4S clusters, exhibit no significant homology to known proteins.

The analysis of nif-lacZYA fusions demonstrated that the genes of R.capsulatus nif region A are organized in seven transcription units. With the exception of the regulatory gene nifA copyI, these R.capsulatus nif operons are preceded by RpoN-dependent consensus promoters (marked by arrows) and by putative NifA-binding sites.

An R.capsulatus mutant carrying a deletion of the structural genes (nifHDK) of the conventional molybdenum nitrogenase is unable to fix nitrogen in the presence of molybdenum but shows slow diazotrophic growth in molybdenum-free medium. This indicates the existence of at least one alternative nitrogenase system in R.capsulatus.



L-40

Improvement of inoculant efficacy by strain improvement and formulation manipulations. Alan S. Paau. Agracetus, 8520 University Green, Middleton, WI 53562, USA.

Rhizobium inoculants have been used in agriculture for many years. As the indigenous population of *Rhizobium* increases, the effectiveness of inoculant use begins to decline. In the US, most *Rhizobium* inoculants are now used on an insurance basis and significant yield increase is rarely obtained.

Many laboratories are now attempting to improve the field efficacy of *Rhizobium* inoculants. Chemical mutants with the ability to fix more nitrogen were first reported in 1978. Since then, many mutants including those with enhanced competitiveness were also reported. Recent advances in molecular biology and the understanding on parameters limiting effective nodulation and nitrogen fixation bring about recombinant *Rhizobium* strains which promise to be more competitive and fix nitrogen more effectively.

Improvement in *Rhizobium* strains alone often fails to improve the field efficacy of inoculant use. Formulations with new properties now promise to complement the advances in strain development. Carrier materials which can be sterilized more easily and coated onto seeds more uniformly have been identified. The use of inorganic carriers also minimizes the stimulation of predator populations in the soil and enhances the nodule occupancy of the inoculant strains. Many additives have also been incorporated into formulations to enhance certain properties of the bacteria and provide supplemental minerals.

The use of improved inoculant strains in combination with new formulation promises to further improve legume yield even in fields that already have high indigenous populations of rhizobia.

L-43

The genetics of actinorhizal *Alnus*. Maurice Lalonde, Centre de recherche en biologie forestière, Faculté de foresterie et de géomatique, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4

Alders (*Alnus* spp.) are ecologically important woody species due to their capacity to fix dinitrogen in association with the filamentous bacteria *Frankia*. Intraspecific and interspecific variations observed in this genus from allozymes and DNA hybridization studies will be reviewed, with emphasis on the Canadian species *A. crispa, A. rugosa* and *A. incana*. Recently, restriction fragment polymorphisms of ribosomal DNA of seven alders confirmed the taxonomy of alder based on morphological characters, by clustering the *Alnus* species tested in each of the two different subgenera *Alnus* and *Alnobetula*. As noted by using isozymes, this RFP study also showed that some alder species are less divergent from the non-actinorhizal species *Betula papyrifera* than from other alder species, suggesting a common ancestor between the lineages *Betula* and *Alnus*. The ability to associate with *Frankia* was probably lost early on in *Betula*. The potential of *Betula* spp. as logical candidates for transfer of host genes that would confer the ability to associate with *Frankia* and form effective dinitrogen-fixing symbiosis will be discussed. Finally, recent advances in the identification of actinorhizal genes and in the development of gene delivery systems for *Alnus* and *Betula* will be briefly presented.

L-44

Recent Studies on stem-nodulating legume- *Rhizobium* symbiosis

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A large area of rainfed lowland rice experiences short-term waterlogging during the 30-50 days dry to wet season transition. This period is too short for upland crops, including grain legumes, even if there were no flooding. However, some aquatic legumes belonging to the genera *Sesbania* and *Aeschynomene*, particularly *S. rostrata* and *Ae. afraspera* are uniquely suited to produce green manure N for the succeeding rice crop during the transition period. Both are fast-growing and root and stem-nodulating.

The symbiont of *S. rostrata*, classified as *Azorhizobium caulinodans*, grows and fixes N_2 in the free-living and N-free condition. Rhizobia of stem-nodulating legumes, *Ae. pratensis*, and *Ae. sensitiva* also possess this ability. These rhizobia also synthesize bacteriochlorophyll under aerobic condition. Consistently observed spontaneous nodulation by *S. rostrata* in the field is attributed to the occurrence of *Az. caulinodans* on seeds, and epiphytically on leaves. *Az. caulinodans* released from stem nodules also survived and grew in flooded soils and the rhizosphere of rice. *S. rostrata* is probably the fastest N_2-fixing plant known. Isotope dilution using ^{15}N-stabilized soil indicated N_2 fixation on the order of 100-240 kg/ha in the dry season and 140-285 kgN/ha in the wet season in 45-55 days. The biological nitrogen fixation contribution ranged from 75-95% of total plant N. Substantial rice yield increases due to green-manuring by *S. rostrata* and *Ae. afraspera* are reported. *Ae. afraspera* has two additional advantages over *S. rostrata* : 1) less photoperiod sensitive, it produces high biomass and N all around year, and 2) it produces profuse branches and less woody stems, which make incorporation easy. The paper discusses on efficient methods for establishment, incorporation, and seed production of *Sesbania* and *Aeschynomene* species, and the research agenda leading to a cost-efficient green manure technology for sustainable rice production.

L-46

KJELD A. MARCKER: REGULATION OF NODULE SPECIFIC GENES.

Kjeld Marcker. Lab. of Gene Expression. Univ. of Aarhus. Dept. of Molecular Biology & Plant Physiology. DK-8000 Aarhus C. Denmark.

1. Identification of regulatory DNA sequences present in nodule specific genes.
   The qualitative and quantitative contributions of four separate cis-acting DNA elements controlling the root nodule specific soybean leghemoglobin lbc3 gene were analyzed in transgenic Lotus corniculatus plants. Expression from internal deletions in the 5' region between positions -49 and -1956 was monitored from a CAT reporter gene. The strong positive element SPE (-1090,-947) responsible for high level expression was, by deleting nodule specific control elements, demonstrated to be an organ regulated element. Deletion of the downstream qualitative organ specific element OSE(-139,-102) containing the putative nodulin consensus sequences 5'AAAGAT and 5' CTCTT resulted in a low expression level. Efficient SPE enhancement is therefore dependent on the organ-specific element which by itself does not enhance expression. This quantitative effect of the immediate upstream region carrying the consensus sequences was also found in hybrid promoter, studies using the soybean nodulin N23 gene promoter, suggesting the involvement of these motifs in a regulatory mechanism for nodulin genes. Deletion of the lbc3 negative element NE (-102,-49) linking the SPE and OSE onto the TATA box did not lead to unregulated expression. These results indicate that interaction between positive, negative and neutral qualitative elements control lbc3 expression.

2. Identification and characterization of nodule specific regulatory proteins involved in soybean N23 and leghemoglobin gene induction.

   Three different organ specific nuclear proteins recognizing AT rich DNA sequences were identified in soybean. One protein (NAT_2) is present in mature nodules, another protein (NAT_1) is detected in roots and nodules, and a third protein (LAT_1) is only observed in leaves. All three proteins recognize several DNA sequences in the promoter region of the nodulin N23 gene. Footprinting, deletion and point mutation analyses reveal different binding properties for all three proteins and further show that even single base pair substitutions have dramatic effects on binding affinity. The LAT_1 and NAT_1 proteins are released from chromation by low salt extraction and are soluble in 2% TCA, indicating a relationship to high mobility group proteins (HMG). DNA binding studies further indicate a functional relationship to the human HMG I protein. Purification and identification of the LAT_1 activity from leaf nuclei, revealed two peptides with molecular weights of 21 kD and 23 kD, respectively, binding the same DNA sequence with equal affinity.

- Dave Emerich: Where does the energy come from?
  - Energy flow to N_2ase.
  - 21 day old plants are optimal
  - C-stress protein. → like heat stress proteins.
  - pattern of proteins after the same.
  - phosphorylation requires $MgCl_2$

- Kjeld Mæller:

  Wood C ≤ 37% DG42 Xenopus leaves.

  $Lbα\ Lbc_1$ $\Psi Lb\ Lbc_3$ — I

  $\Psi_2 Lb\ Lbc_2$ — II

  stay tuned on Lbc_3

- Carroll Vance:
  - AAT 1/2 cross-reacts with soybean AAT 1/2. Use in Western.
  - cloned AAT-2 (45% homology to AAT from animal mitochondria)
  - no specific PEPC in nodules
  - selection of high + low enzyme variants in cell resulted in a nodule mass compensation in both directions.

L-47

Alfalfa root nodule aspartate aminotransferase (AAT): Biochemical importance and genetic control. C.P. Vance. USDA-ARS Plant Science Research, Dept. of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA.

Aspartate aminotransferase (AAT; EC 2.6.1.1.) catalyzes the biosynthesis of aspartate from glutamate and oxaloacetate. The enzyme plays an essential role in: 1) the assimilation of fixed N into asparagine in legume root nodules; 2) the transfer of fixed carbon from mesophyll cells to bundle sheath cells in C_4 plants; and 3) the flow of reducing equivalents to chloroplasts and mitochondria through a malate-aspartate shuttle. Alfalfa root nodule AAT is comprised of two major forms, AAT-1 and AAT-2. Root nodule AAT activity increases during effective nodule development. This increase in total activity is associated with an increase in AAT-2 activity and enzyme protein. The absolute amount of AAT-2 increased during nodule development from 0.023 to 0.373 μg AAT-2·mg fw tissue^{-1} (a 16-fold increase). By contrast, AAT-1 appears to be more closely associated with roots. We have purified both forms of AAT to near homogeneity and produced high titre polyclonal antibodies to the native and subunit proteins. Alfalfa AAT-1 was purified from root suspension culture cells, while AAT-2 was purified from root nodules. Both enzymes appear to be homodimers, with AAT-1 having a native and subunit MW of 88±5 and 42±2 kd respectively. Corresponding MWs for AAT-2 were 80±3 and 40±2 kds, respectively. Immunological analysis showed that AAT-1 and AAT-2 are very distinct. Anti-AAT-1 serum did not recognize AAT-2 and conversely, anti-AAT-2 serum showed no recognition of AAT-1. Such data indicates that the amino acids comprising the various antigenic epitopes of AAT-1 and AAT-2 are strikingly different and suggests that the isozymes are encoded by separate genes. Antibodies to alfalfa nodule AAT-2 recognized a comparable 40 kd polypeptide from nodules of several legume species and leaves of both C_3 and C_4 species. About 90% of the total AAT activity of alfalfa nodules was comprised of AAT-2, with the remaining 10% comprised of AAT-1. Root total AAT activity of most species tested was comprised mainly of AAT-1. In effective nodules AAT-2 made up about 2% of the total soluble protein. The absolute concentration of AAT-2 in effective nodules was 7-fold that of ineffective nodules. Genetic variation for total AAT activity was evident in several alfalfa germplasms. Bidirectional selection was successful in generating alfalfa populations having high and low root nodule AAT activity. Changes in total nodule AAT activity due to selection resulted from selectively increasing or decreasing AAT-2 enzyme protein. A 1.2 kb AAT-2 cDNA clone was selected from an immunoscreen of an alfalfa nodule cDNA library prepared in λgtll.

L-48

Regulation of host gene expression during nodule development in soybeans. C. Sengupta-Gopalan. Dept. of Agron. & Hort./Plant Genet. Eng. Labs., New Mexico State Univ., Las Cruces, NM 88003, USA.

The establishment of the rhizobia-legume root nodule and its subsequent development leading to symbiotic N_2-fixation, involves participation from both partners. Several bacterial and host genes are specifically expressed at different stages of nodule development. Our paper will focus on soybean genes that are expressed in both the early and late stages of symbiosis.

To understand the early stages of symbiosis, we have focused on the expression of two specific gene families known to be involved in symbiotically important processes such as synthesis of isoflavonoid signal molecules and host defense molecules in the form of isoflavanoid phytoalexins. These are phenylalanine ammonia lyase (PAL) which catalyzes the first step in the general phenylpropanoid biosynthetic pathway and chalcone synthase (CHS) which catalyzes the first step in flavanoid and isoflavanoid biosynthesis. We have isolated and characterized cDNA clones for CHS and PAL from a cDNA library to soybean nodule RNA and compared the sequence with published sequences for the corresponding genes from bean and parsley. Using different approaches, the expression of CHS and PAL genes, in effective and ineffective symbiosis and pathogensis, has been studied and will be discussed.

As a first step towards understanding regulation of the late nodulin genes, we have categorized them into distinct classes of coordinately expressed mRNA. Furthermore, genes for several of the more abundant nodulins in soybean, can be categorized based on localized sequence conservation in the coding regions of the genes. Two of these gene families which also belong to two different temporal classes of mRNA, have been selected for detailed analysis. We have isolated and characterized cDNA and genomic clones for several of these gene families. The characterization of cDNA and genomic clones belonging to these two gene families include: i. Nucleotide sequencing and alignment of highly conserved domains; ii. Their hybridization pattern to nodule RNA; iii. Southern analysis of genomic DNA; iv. SDS-PAGE and 2D gel electrophoresis of translation products of nodule RNA, hybrid selected with cloned DNA for the different members. Based on these results, we predict the possible functional role of the corresponding nodulins, the organization of the genes in the genome and the phylogenetic relationship among the members.

To understand the basis for the differential temporal accumulation of mRNA for the two families, the promoter regions of a representative gene from each family has been analyzed. Promoter analysis involves identification and characterization of 'cis' acting elements and 'trans' activating factors that bind to promoter elements in a specific manner. The results of this analysis will be discussed.

L-50

An hypothesis for the evolution of actinorhizal symbioses. B. C. Mullin, S. Swensen and P. Goetting-Minesky. The Botany Department, The Plant Physiology and Genetics Program and the Center for Legume Research. The University of Tennessee, Knoxville, TN 37996, USA.

Actinorhizal symbioses involve symbiotic relationships between actinomycetes of the genus Frankia and host plants belonging to 24 genera distributed among eight higher plant families. Although all host plant species are woody dicots, there is no close phylogenetic affinity among host plant groups. Nor is there agreement among actinomycete biologists as to whether host plants have evolved from a common nodulated ancestor or whether symbiosis has arisen independently more than one time resulting in the current diversity of host plant species. Biogeographical, paleontological, phylogenetic and physiological data will be presented in support of the latter hypothesis and a plan to further test this hypothesis will be presented.

L-51

Genetics of Alternative Nitrogen Fixation Systems in *Azotobacter vinelandii*

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A. vinelandii is able to fix N_2 by any of three genetically distinct nitrogenases. Besides nitrogenase-1, the well characterized Mo-containing nitrogenase, this diazotroph harbors 2 Mo-independent nitrogenases; nitrogenase-2, a V-containing enzyme, and nitrogenase-3, an enzyme that does not appear to contain either Mo or V. Each of the three enzyme complexes is composed of 2 components; dinitrogenase reductase and dinitrogenase. The nucleotide sequence is known for the structural genes encoding the subunits of each of the 3 nitrogenases. *nifH*, *vnfH*, and *anfH* encode the subunits making up dinitrogenase reductases-1, -2, and -3, respectively, while *nifDK*, *vnfDK*, and *anfDK* encode the α and ß subunits of dinitrogenases-1, -2, and -3. Dinitrogenases-2 and -3 each contain a newly discovered subunit (δ) encoded by *vnfG* and *anfG*. The organization of the structural genes is: promoter-*nifHDK*; promoter-*vnfH*; promoter-*vnfDGK*; and promoter-*anfHDGK*. Each operon contains promoters that conform to the RNA polymerase-sigma 54 recognition sequence. Proteins encoded by *nifA*, *vnfA*, and *anfA* are required for expression of nitrogenases-1, -2, and -3, respectively. Upstream activating sequences, however, have not been found in the promoter regions of the *vnf* and *anf* operons. Transcription of the *anfHDGK* operon also requires the *vnfH* gene product (dinitrogenase reductase-2).

nifENX-like ORFs are located immediately downstream from *vnfA*. Preliminary results with a strain containing Tn*5*-*lacZ* inserted towards the 3' end of the *nifN*-like ORF indicate that the *nifN*- and/or *nifX*-like ORF is required for diazotrophic growth in Mo-deficient media with or without V. However, in the presence of Mo this strain shows wild-type growth. These results imply that these *nifENX*-like genes are required for functional nitrogenases-2 and -3 but not nitrogenase-1.

The predicted amino acid sequences for the *nifH*, *vnfH*, and *anfH* gene products exhibit more similarity amongst each other (63-91% identity) than those for the α and ß subunits of the 3 different dinitrogenases (31-57% identity). The *vnfDK* and *anfDK* genes are more similar to each other than either set of genes is to *nifDK*. The percentage identity between amino acid sequences of the α and ß subunits of each nitrogenase (19.8-24.6%) suggests that the genes encoding these two subunits may have evolved from a common ancestral gene as previously suggested for Mo-containing nitrogenases. Based on identity comparisons it has been speculated that *nifD* and *nifK* may have diverged somewhat earlier during evolution than genes encoding the subunits of nitrogenases-2 and -3.

L-52

Nitrogen fixation in filamentous cyanobacteria. R. Haselkorn. Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

Cyanobacteria have the same requirements for nitrogen fixation as other diazotrophs: synthesis of the nitrogenase complex and its cofactors, a source of ATP and low potential electrons, and an environment protected from oxygen. Unique among diazotrophs, cyanobacteria evolve oxygen in the light. Filamentous strains such as Anabaena solve the problem of oxygen protection of nitrogenase by differentiating specialized cells called heterocysts at regular intervals along the filament. In the heterocyst, the oxygen-evolving reaction of Photosystem II is turned off, CO_2 fixation stops, nitrogenase, cofactors and electron donor proteins are synthesized, and the oxidative pentose pathway is activated. A double-layered envelope of polysaccharide and glycolipid is laid down outside the existing cell wall. New connections between the heterocyst and the adjacent vegetative cells are established; these mediate the transport of carbohydrate and of fixed nitrogen. All of these changes require differential gene expression.

The major vegetative cell RNA polymerase has been purified from Anabaena 7120. The σ factor, which controls promoter recognition, is similar to that of eubacteria. The promoters of genes expressed in Anabaena vegetative cells are recognizable eubacterial promoters. Genes expressed uniquely in heterocysts have different promoters and they are not transcribed in vitro by purified vegetative cell RNA polymerase. Attempts to identify a new σ factor required for expression of heterocyst-specific genes, at the level of both gene and protein, have been unsuccessful.

The organization of the genes for nitrogen fixation in Anabaena was originally thought to be very different from that of Klebsiella and other diazotrophs. Subsequently it was discovered that the vegetative cell DNA of Anabaena 7120 carries two interrupting elements: an 11-kb element inserted near the carboxy terminus of the nifD gene and a 55-kb element inserted near the nifS gene. Both elements are flanked by short directly repeated sequences within which conservative site-specific recombination occurs during heterocyst differentiation. The result of these recombinations, in each case, is precise excision of the element and fusion of the heterocyst chromosome. In the case of the 11-kb element, excision leaves behind the intact nifHDK operon. The other rearrangement yields the nifB fdxN nifSU operon, where nifBSU have the same significance as their Klebsiella counterparts and fdxN encodes a bacterial ferredoxin-like protein of unknown function. There is a heterocyst-specific plant-like ferredoxin that has been shown to donate electrons directly to nitrogenase in Anabaena extracts; this ferredoxin is encoded by the fdxH gene mapping downstream of nifK. Between fdxH and nifK there are at least six open reading frames, some of which are similar to Klebsiella nif genes.

A large number of mutants of Anabaena 7120, defective in heterocyst differentiation, have been isolated. For some of these, the wild type gene that complements the mutation has been isolated by conjugation with a cosmid library. It should be possible to find, among these genes, those that encode components of the regulatory cascade leading to heterocyst differentiation, as well as structural genes for glycolipid and polysaccharide synthesis and for the establishment of connections between heterocysts and vegetative cells.

L-53

Biochemical Genetic Analysis of the Nitrogenase MoFe Protein.

Dennis. R. Dean, Department of Anaerobic Microbiology, The Virginia Polytechnic Institute and State University, Blacksburg, Virginia

Metallocluster extrusion requirements, interspecies MoFe protein primary sequence comparisons, comparison of MoFe protein α- and β-subunit primary sequences to each other, and comparison of the MoFe protein subunits to products of genes required for FeMoco biosynthesis (*nifE* and *nifN*), have been used to formulate a model in which the metallocluster domains present within the *Azotobacter vinelandii* MoFe protein are assigned. The purpose in developing such a model is to provide a rational basis for examining the functional properties of the individual metallocluster types and to determine the contribution of the polypeptide environment to those functions. Our model has the following salient features: 1) Regions of primary sequence conservation surrounding conserved α-subunit Cys residues 62, 88 and 154; and β-subunit Cys residues 70, 95 and 153 are targeted as P cluster environments; 2) P cluster domains are equally distributed among the MoFe protein α- and β-subunits; 3) Individual pairs of P clusters are located close to each other or form a larger cluster bridged between the MoFe protein α- and β-subunits (we favor the latter possibility); 4) P clusters are solvent exposed or are close to the polypeptide's surface; 5) Regions surrounding MoFe protein α-subunit Cys residues 183 and 275 provide major FeMo-cofactor domains.

We have begun to test this model and to probe the functional properties of the assigned P cluster and FeMoco environments by using site-directed mutagenesis. The following approaches have been used: 1) A variety of different amino acids were substituted for those residues targeted as potentially providing direct metallocluster ligands. Emphasis was placed on conserved Cys and His residues contained within the MoFe protein α-subunit; 2) Certain residues within proposed FeMoco domains located within the α-subunit were substituted by the corresponding residues from the *nifE* gene product; 3) Certain residues within the *nifE* gene product were substituted by the corresponding MoFe protein α-subunit residues; 4) Certain residues within a proposed FeMoco domain located within the α-subunit were substituted by the corresponding residues from the *C. pasteurianum* sequence. All of these mutant strains have been analyzed for their acetylene reduction activities, EPR spectra, and diazotrophic growth properties. Certain mutant strains have also been analyzed by ESEE spectroscopy. In addition, mutations resulting in known amino acid substitutions within the MoFe protein were also placed in combination with *nifB*-, *nifE*-, *nifN*- or *nifV*-deletion mutations. Biochemical reconstitution experiments involving extracts prepared from such double mutant strains were used to establish the contribution of certain known amino acid substitutions to observed alterations in the S = 3/2 EPR signal and in substrate reduction patterns. Results from site-directed mutagenesis studies described above will be summarized and their relevance to our metallocluster domain assignments will be discussed.

Experiments that will be described in this talk were performed in collaboration with Kevin Brigle, Robert Setterquist, Debbie Scott, Hal May, Hans Thomann, and William Newton.

L-54

Nitrogen Fixation Genes of Clostridium pasteurianum.

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Clostridium pasteurianum (Cp) is a Gram-positive, obligately anaerobic N_2-fixer. Major nitrogen-fixation (nif) genes of C. pasteurianum have been cloned and sequenced. In a region spanning 11.6 kb of DNA, we have identified genes that correspond to nifH, nifD, nifK, nifE, nifN, nifB, and nifV of Klebsiella pneumoniae and Azotobacter vinelandii. The genes of C. pasteurianum have been designated nifH1, D, K, E, N-B, Vω, Vα, which occur in this order, with nifN-B and nifVω being separated by two other open reading frames (ORFs). Identification of these genes was based on (i) an agreement between the predicted amino acid sequence of the gene and the complete or partial amino acid sequence of the protein (nifH1, nifD, and nifK), (ii) a similarity in the predicted amino acid sequence of the gene with that of the well characterized genes of other organisms (nifE, nifN-B, nifVω, and nifVα), and (iii) the complementation of a specific genetic defect in A. vinelandii (nifVα and nifVω). There are several unique features in the organization or structure of the Cp nif genes: (i) structural genes for nitrogenase and genes for the synthesis of the FeMo-cofactor occur in close proximity, (ii) the stop codon for nifD and the start codon for nifK overlap, (iii) one gene (nifN-B) encompasses both nifN and nifB, and (iv) two genes (nifVα and nifVω) together encode the nifV polypeptide of A. vinelandii and K. pneumoniae. In a 17.5 kb region that includes the above described 11.6 kb of DNA, there are also a nifH-like sequence (nifH2, which is upstream to nifH1) and a total of five ORFs with unknown functions. In addition, four additional regions, each of which contains a nifH-like sequence (nifH3 through nifH6), were found. Among nifH1 and the nifH-like sequences, the predicted amino acid sequence of nifH3 differed most from the others. Transcripts for the nifH-like sequences, except for nifH3, have been detected in N_2-fixing cells grown under molybdenum-sufficient conditions. The predicted amino acid sequences of nifH3 of C. pasteurianum and anfH of A. vinelandii have an 82% similarity, but it remains to be shown if Cp nifH3 encodes an alternative nitrogenase. The transcription start site for transcription units containing, respectively, nifH1, nifE, nifH2/H6, nifH4, and nifH5, has been determined. Nucleotide sequences upstream from the transcription start sites do not contain the conserved nif promoter and upstream activator sequences found in K. pneumoniae and other Gram-negative organisms. However, a common sequence occurs in the -100 region and another occurs in the -35 and -10 regions of the nif transcription start sites of C. pasteurianum.

L-55

"The genetics and biochemistry of the reversible ADP-ribosylation systems of *Rhodospirillum rubrum* and *Azospirillum lipoferum*".
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In addition to the various levels of transcriptional and post-transcriptional regulation governing the synthesis of *nif* gene products, a number of bacteria employ a post-translational regulatory system that regulates the activity of the nitrogenase complex. In a number of these cases, the regulation occurs by the reversible covalent modification of the dinitrogenase reductase dimer by a single molecule of ADP-ribose. The modification reaction s performed by dinitrogenase reductase ADP-ribosyl transferase DRAT, encoded by *draT*), while the removal of the ADP-ribose is performed by dinitrogenase reductase activating glycohydrolase (DRAG, encoded by *draG*), and a substantial body of work exists on the *in vitro* characterization of these two enzymes. The best studied organism with this regulation is *Rhodospirillum rubrum*, where the both enzymes, as well as their substrate, have been purified, and the genes encoding all three have been identified, sequenced, and mutationally analyzed. Expression vectors containing *draTG* have been constructed and introduced into *Klebsiella pneumoniae*, where the proteins are not only synthesized, but produce a reversible regulation of nitrogenase in response to nitrogen status that is superficially quite similar to that seen in *R. rubrum* itself. These varied approaches have allowed us to conclude that the *draT* and *G* gene products are both necessary and sufficient for reversible ADP-ribosylation and that each of these proteins is itself subject to some form of post-translational regulation of activity. The analysis of *R. rubrum* strains containing mutations in *draT* and *G* also allows the conclusion that DRAG and DRAT are the enzymes responsible for the *in vivo* ADP-ribosylation system. The regulation of *K. pneumoniae* nitrogenase activity by the *R. rubrum* proteins has been reproduced with the cloned *draTG* genes from *Azospirillum lipoferum*. These results with heterologous gene expression suggest either that the DRAG and DRAT proteins themselves sense and respond to nitrogen status or, more likely, they are able to interact with functionally conserved *K. pneumoniae* proteins that sense nitrogen status. Transcriptional studies in *R. rubrum* suggest that the transcription of the *dra* region is complex and is regulated in response to a variety of growth conditions.

L-56

The First Photosynthetic N_2-Fixing Rhizobium: Characteristics. Allan R.J. Eaglesham[1], Joan M. Ellis[1], William R. Evans[2], Darrell E. Fleischman[2], Mariangela Hungria[1], and Ralph W.F. Hardy*[1].
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The characteristics of the first photosynthetic rhizobium to be described will be presented and the name Photorhizobium thompsonianum is proposed to distinguish it from the other rhizobial genera of Rhizobium, Bradyrhizobium, Azorhizobium, and Sinorhizobium. A rhizobial strain BTAi 1, isolated several years ago from West Virginia sand by Eaglesham at Boyce Thompson Institute and shown to form N_2-fixing stem and root nodules in Aeschynomene indica, is reported now to be photosynthetic and N_2-fixing in free-living culture. Colonies of BTAi 1, grown aerobically on appropriate medium in a dark-light cycle at Battelle-C.F. Kettering Laboratory, were found to be pink-red in color and contain modest amounts of Bacteriochlorophyll a, comparable to those found for marine aerobic photosynthetic bacteria.

Functional evidence of photosynthesis by free-living BTAi 1 includes (1) positive effect of light on viable cell count during the stationary growth phase, (2) light stimulation of CO_2 uptake, (3) light decrease in O_2 uptake, and (4) photosynthetic inhibitors and uncouplers overcoming the above light effects on CO_2 and O_2 uptake. Evidence of N_2-fixation by free-living BTAi 1 and of a functional relationship between photosynthesis and N_2 fixation includes (1) reduction of acetylene to ethylene in a N-free agar medium with decreased pO_2, (2) a slow, 2-5 fold increase in acetylene reduction activity response to light in a carbon-limited medium, and (3) increase of cell protein content with a light-dark cycle versus continuous dark.

Rhizobia that form stem nodules on Sesbania rostrata are aerobic, fast growing, fix N_2 as free-living cultures in the absence of exogenous N, and are not photosynthetic; they have recently been assigned to the new genus Azorhizobium. BTAi 1 is aerobic, fast growing, fixes N_2 in free-living culture based on acetylene reduction to ethylene in the absence of exogenous N, is photosynthetic, and forms stem nodules on A. indica where N_2-fixing activity is enhanced by long-wavelength light. The symbiotic activities of BTAi 1-Aeschynomene, including specific N_2-fixing activity, will be compared with those of Azorhizobium-Sesbania. We propose the genus name Photorhizobium and the species name thompsonianum for BTAi 1. It is suggested that stem nodules with photosynthetic rhizobia may be more energy self-sufficient for N_2 fixation than root nodules or stem nodules containing non-photosynthetic rhizobia.

L-57

Nodulation of Non-legumes by Rhizobia.

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Our finding that brief treatment of roots of clover seedlings with cell wall degrading enzymes removed a barrier to Rhizobium-host specificity (Al-Mallah, Davey and Cocking, Bio/Technology (1987), 5, 1319) led us to investigate whether a similar enzymatic treatment, already known to expose the plasma membrane at the tips of both legume and non-legume root hairs (Cocking, Bio/Technology (1985), 3, 1104), would enable enzyme-treated non-legume seedlings to be effectively nodulated by rhizobia.

Nodular structures were induced on both rice and wheat roots (Al-Mallah, Davey and Cocking, J. Exp. Bot. (1989), 40, 473; Int. J. Genetic Manipulation in Plants (1989), 5,1) by treating roots of seedlings with a cell wall degrading enzyme mixture (1% cellulase and 0.1% pectolyase in 8% mannitol) followed by inocualtion with rhizobia in the presence of polyethylene glycol. Rhizobia were located both within and between cells of the nodular structures, but no nitrogenase activity was detectable using the acetylene reduction procedure.

This finding then led us to investigate whether the non-legume, oilseed rape (Brassica napus), taxonomicaly more closely related than rice or wheat to the non-legume Parasponia, the only non-legume nodulated naturally by rhizobia, would be more responsive than rice and wheat to nodulation by rhizobia using this enzyme-PEG treatment. Using this treatment we have recently observed the production on oilseed rape seedlings of nodules with close similarities, both morphologically and structurally, to legume nodules, and with low nitrogenase activity.

These results are encouraging in terms of the goal of developing a nitrogen-fixing symbiotic association between naturally occurring rhizobia and non-legumes. These results are also of interest in relation to the evolution of nitrogen-fixing symbiotic associations.

Discussion session (1):

① nodO protein – some homology, → nodO⁻ = high BNF, some NN
– some binding to plant membrane.

② Geiger: 4' phosphopantetheine group with nodF
NCP ↳ nodE

③ F. Dazzo: some effect of nod genes on EPS decoration.
– caution: effect of acetyl transferase or phosphotransferase.

Alan Downie: no effect of nod gene induction on EPS (with Dudman)

Fauke: fixR = NAD dehydrogenase homology.

SESSION A

Recent advances in the chemistry of nitrogen fixation

A-01 - A-23

A-01

Comparison of the MoFe Protein from Wild Type and FeMo cofactor-Deficient Mutants of Azotobacter vinelandii. S. Tal*; T.W. Chun; J.- G. Li; S. Kim and B.K. Burgess. Department of Molecular Biology and Biochem., Univ. of California, Irvine.

The MoFe proteins from various Azotobacter vinelandii FeMo cofactor (FeMoco)-deficient mutants were compared to that of the wild-type (WT) strain in terms of reconstitution of the MoFe protein activity; stability to heat treatment; appearance on anaerobic native gel electrophoresis and partial purification. The strains compared were: DJ54 (Δ*nifH*), which does not synthesize the Fe protein or FeMoco; DJ71 (Δ*nifV*), which has been proposed to have a mutation in the homocitrate biosynthesis pathway; DJ35 (Δ*nifE*) and DJ141 (Δ*nifB*), both of which have mutations in the FeMoco biosynthetic pathway. It was found that the addition of isolated FeMoco to mutant extracts restored DJ54 MoFe protein activity to WT levels, but restored DJ35 and DJ141 MoFe protein activity to only half that of WT. DJ71 has higher MoFe protein background activity, which increases slightly by the addition of FeMoco. In addition it was found that DJ54 MoFe protein lost as much as 90% of its activity as a result of heat treatment (5 min at 56°C), whereas the MoFe protein activity from the other mutants remained the same after heat treatment. Native anaerobic gel electrophoresis reveals three different patterns for the mutants. WT and DJ71 show only one band, which reacts with anti-MoFe protein antibodies. DJ35 and DJ141 show two bands cross-reacting, one in the position of the WT band and one below, while DJ54 shows one cross-reacting band above the position of the WT band. Purification results will be presented.

A-02

X-Ray absorption spectro-electrochemistry of the nitrogenase iron-molybdenum cofactor. J.S. Cantwell*[1], W.E. Newton[1], B.J. Feldman[1], S.F. Gheller[1], F.A. Schultz[2], P. Frank[3], B. Hedman[3], and K.O. Hodgson[3], USDA-ARS-WRRC, Albany, CA 94710, Chemistry Dept., Indiana Univ.-Purdue Univ., Indianapolis, IN 46223, and Chemistry Dept., Stanford University, Stanford, CA 94305.

Enzyme-bound iron-molybdenum cofactor (FeMoco) is the active catalytic center of nitrogenase. Like its protein-bound form, isolated FeMoco after extrusion into N-methylformamide, NMF undergoes two redox processes. These interconvert the semi-reduced, EPR-active form with either the more oxidized, EPR-silent form or the fully reduced, EPR-silent form. When protein bound, this last form is responsible for nitrogen fixation. We have built an anaerobic electrochemical cell compatible with XAS measurements at 2.4-3.0 KeV to study FeMoco at the sulfur K and the Mo L_2 and L_3 edges, which are sensitive to changes in electronic and structural environment, in all three redox states. In addition to finding unprecedented ligation by thiosulfate, plus the expected sulfide, these measurements indicate that Mo is not involved in either of FeMoco's redox couples, which suggests Fe and/or S as its major redox components.

A-03

Evidence for cysteine ligation to a metal at site 153 in the K gene of nitrogenase MoFe protein in *Azotobacter vinelandii*.

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The *a* and *b* subunits of the nitrogenase MoFe protein are encoded by *nifD* and *nifK* respectively. Site-directed mutagenesis of a cysteine to alanine (C to A) at site 153 in the K gene resulted in a Nif- phenotype. Substitution of the cysteine with serine (C to S) or deletion of the cysteine (ΔC) resulted in a Nif+ phenotype but a slowed grwoth rate. The MoFe protein acetylene reduction activity in crude extracts of the C to S and ΔC mutants was approximately 50% of wild type activity. Maximum ammonia, hydrogen and ethylene production by purified C to S MoFe protein was 40% of wild type, however the mutant activity equalled that of wild type when electron flux was diminished by a lowering of the Fe protein to MoFe protein ratio. The change in mutant MoFe activity versus wild type activity as electron flux increases suggests that the K153 ligand may be involved with an intermediate electron donor/acceptor in the protein, e.g. an Fe/S cluster.

A-04

Evidence for FeMo cofactor association with the nitrogenase *nifD* gene product in *Azotobacter vinelandii*.

D.J. Scott*, W.E. Newton, K.E. Brigle, and D.R. Dean. USDA, ARS-WRRC, Albany, CA 94710 and Dept. Anaerobic Microbiology, Virginia Polytechnic Institute, Blacksburg, VA 24061

The MoFe protein of nitrogenase consists of 2 *a* and 2 *b* subunits which are encoded by *nifD* and *nifK*, respectively. A strategy has been developed for the site-directed mutagenesis of the *Azotobacter vinelandii nifD* gene. Using this strategy, we have isolated two mutants with altered MoFe *a* subunits, each containing a single amino acid change. The change was dictated by comparison of conserved interspecies amino acid sequences of MoFe subunit and the FeMo cofactor biosynthetic gene, *nifE*. The phenotype of these two mutants was Nif-. However, unlike the native nitrogenase, the crude extracts of both mutants produced ethane, in addition to ethylene, from acetylene. The whole cell EPR signal of each mutant was individually altered from the wild type cells. These results indicate that the *a* subunit interacts with the FeMo cofactor, which is located at the substrate-reducing site.

A-05

Dinitrogen reduction by dinitrogenase activated *in vitro* is dependent upon which diastereomer of 1-fluorohomocitrate is incorporated into the iron-molybdenum cofactor. M. Madden*, V. Shah, N. Kindon, S. Burke, and P. Ludden. Dept. of Biochem. University of Wisconsin, Madison, WI. 53706, USA.

Homocitrate has been shown to be an integral part of the iron-molybdenum cofactor (FeMo-co) of dinitrogenase where one mole of homocitrate is found for each mole of molybdenum. The *in vitro* FeMo-co synthesis system allows the incorporation of homocitrate and its analogs into FeMo-co which show altered substrate specificities (N_2, C_2H_2, or H^+ reduction) of nitrogenase. We have found that dinitrogenase activated *in vitro* using *threo* (1R,2S)-fluorohomocitrate reduces protons and C_2H_2, but cannot reduce N_2. Proton reduction is inhibited by carbon monoxide, a characteristic of the nitrogenase from $NifV^-$ mutants. Dinitrogenase activated *in vitro* using the *erythro* (1S,2S)-fluorohomocitrate reduces protons, C_2H_2, and N_2. In this case proton reduction is not inhibited by CO, as is characteristic of the wild type enzyme. Cyanide reduction properties of dinitrogenase activated with various homocitrate analogs will also be presented. ^{19}F-NMR spectroscopy of partially purified dinitrogenase activated with each diastereomer of 1-fluorohomocitrate shows a very broad signal (>20 Hz) shifted some 80 ppm from the doublets typically observed with the free acid forms. Finally, carbonyl sulfide (COS), an analog of CO, inhibits proton and C_2H_2 reduction by dinitrogenase to varying extents, dependent upon the analog of homocitrate incorporated into the iron-molybdenum cofactor.

A-06

Kinetic and spectroscopic studies on the interactions between nitrogenase proteins, substrates, products and inhibitors. D.J. Lowe*, G. Ashby, K. Fisher, B.D. Howes and R.N.F. Thorneley. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

The interactions between Klebsiella pneumoniae molybdenum nitrogenase proteins, acetylene, its reduction product ethylene, and a number of analogues have been studied by a variety of techniques. These include both steady-state and pre-steady-state kinetic methods as well as e.p.r. and ENDOR. The results will be discussed in terms of the chemistry of alkyne and alkene binding to transition metal centres, and of the structure and mechanism of action of nitrogenase.

A-07

Kinetic studies on the mechanism of ATP-hydrolysis by Klebsiella pneumoniae nitrogenase. R.N.F. Thorneley*†, G.A. Ashby†, C. Julius†, J. Hunter†† and M.R. Webb††. †AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K. and ††MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, U.K.

Stopped-flow spectrophotometry[1], stopped-flow calorimetry[1], steady-state H_2 and P_i formation assays and ^{18}O-loss from ^{18}O-P_i with mass spectrometric analysis[2] have been used to investigate the mechanism of reductant-dependent and reductant-independent ATP hydrolysis by purified nitrogenase component proteins at 6°C, pH 7.0 and at 23°C, pH 7.4. MgATP cleavage to yield nitrogenase bound MgADP+P_i is reversible, coupled to the release of a proton, endothermic and precedes electron transfer from the Fe- to the MoFe-protein at 6°C. Further evidence for reversible cleavage of MgATP comes from MgADP catalysed loss of ^{18}O from ^{18}O-P_i under conditions of reductant-independent ATPase activity at 23°C.[3]

1 R.N.F. Thorneley, G.A. Ashby, J.V. Howarth, N.C. Millar and H. Gutfreund (1989) Biochemical Journal 264, 657-661.
2 M.R. Webb, M.G. Hibberd, Y.E. Goldman and D.R. Trentham (1986) Journal of Biological Chemistry 261, 15557-15564.
3 C. Julius (1990) Diplomarbeit Thesis, Univ. Köln, W. Germany.

A-08

Genetic and Biochemical Analysis of the Roles of *K. pneumoniae nif* Genes in the Production of the apoMoFe Protein of Nitrogenase.

T.C. White*, G. Harris, J. Flory and W.H. Orme-Johnson, from the Dept. of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139 USA

Systematic deletions of individual nitrogen fixation *(nif)* genes from a binary plasmid system used to produce *K. pneumoniae* nitrogenase components in *E.coli* showed that the minimum set of *nif* genes required for maximal production of the iron-molybdenum cofactor (FeMoco) reactivatable apo-molybdenum-iron (apoMoFe) protein of nitrogenase is *nifHDKTYUSWZM+A* (pVL222+pVL15). This apoMoFe protein is identical to that produced by a FeMoco deficient mutant of *K. pneumoniae* in its ability to be reactivated by FeMoco and in its electrophoretic mobility on anaerobic non-denaturing gels. Addition of FeMoco to both apoMoFe proteins increased their electrophoretic mobilities, in a two-step fashion, to that of holoMoFe protein on these native gels. Deletion of *nif* genes, individually or in groups, from pVL222 resulted in partial or complete loss of apoMoFe activity. ^{55}Fe-radiolabel and immunodetection techniques showed that several of these gene products are involved in the assembly of the unique Fe_4S_4 P-clusters of the apoMoFe protein, whose subunits are encoded by *nifDK*. Deletion mutants deficient in apoMoFe protein activity produce an immunoreactive tetrameric species comigrating with active apoMoFe protein which contains no detectable iron and does not undergo this same FeMoco-induced two-step mobility shift on native gels. SDS-PAGE analysis of the mutant extracts, as well as those prepared from *in vivo* ^{35}S-pulse labeled cultures, provided insight into the interaction of the *nif* genes on pVL222 which process NifD and K into apoMoFe protein.

This work supported by the National Institutes of Health under GM30943.

A-09 BIOSYNTHESIS OF THE IRON-MOLYBDENUM COFACTOR OF NITROGENASE. Anne-Frances Miller* & William. H. Orme-Johnson, Dept. of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139 USA.

The iron-molybdenum cofactor (FeMoco) is believed be a critical component of the catalytic site of nitrogenase. An understanding of the mechanism of assembly of this cofactor should provide important insights into the structure of finished FeMoco and the means by which FeMoco's production and final form are controlled biochemically. The assembly of FeMoco is mediated by the products of at least six nitrogen fixation *(nif)* genes. Of these, we have cloned the *nif* genes *E* and *N* from *Klebsiella pneumoniae* into an over-production vector and observed proteins corresponding to each of these by polyacrylamide gel electrophoresis. Furthermore, we have developed a binary plasmid system using *nif* genes from *K. pneumoniae* that supports synthesis of FeMoco and active molybdenum-iron (MoFe) protein in *Escherischia coli*, at 10 - 20 % the level of production by wild-type *K. pneumoniae*. We are currently extending this system to study the mechanism of FeMoco assembly in *E. coli* by electron paramagnetic resonance (EPR) spectroscopy. EPR spectra of MoFe protein produced in *E. coli* from *K. pneumoniae nif* genes will be presented.

A-10 PHYSICAL CHARACTERIZATION OF THE MOLYBDENUM-IRON COFACTOR OF NITROGENASE. Thomas A. Collet, Alison B. Hickman, Thanos Salifoglou, David W. Wright, and William H. Orme-Johnson. Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

The molybdenum-iron protein of nitrogenase contains four atypical Fe_4S_4 clusters and two molybdenum-iron cofactors (FeMoco) of approximate metal composition $MoFe_{5-8}S_{8-10}$ which are believed to be the sites of N_2 binding and reduction to NH_3. Recently, homocitrate (2-hydroxy-1,2,4-butanetricarboxylic acid) has been shown to be an organic moiety of FeMoco. A new protocol has been developed in our laboratory enabling us to extract intact FeMoco from the MoFe protein into various organic solvents in a quantity and purity suitable for novel analytic investigation. By applying the technique of X-ray photoelectron spectroscopy (XPS) to FeMoco and various FeS- and MoFeS-containing model compounds, we have developed a model for the elemental composition, overall charge, and Mo oxidation state of the cofactor.

Physical experiments and EPR spectroscopy have been used to determine the stability of FeMoco in a number of organic solvents. Homocitrate and citrate are also under investigation in order to determine their roles in the structure and catalytic function of FeMoco.

A-11 Maturation of the Iron Protein of *K. pneumoniae* and Relevance of Conserved Amino Acid Residues for its Activity and Stability.

T.A. Collet*, T. White, K. Howard†, M.P. Reeve, and W.H. Orme-Johnson, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA, †Proctor and Gamble, Cincinnati, OH 45241, USA.

A binary plasmid system is used to produce the iron protein of *K. pneumoniae* under the control of its native promoter in *E. coli*. Sequence analysis of the plasmid insert in conjunction with deletion analysis reveals that the *nif*M gene product together with *nif*H is necessary and sufficient for the production of active iron protein. ^{55}Fe labeling in conjunction with immunological analysis shows that *nif*M gene product is responsible for the insertion of the Fe_4S_4 cluster into the apo-Fe protein, which could be detected by native gel electrophoresis. Immunological data on *nif*M and two gene products related to it - *nif*Z and *nif*W - will be discussed.

The amino acid sequence of the iron protein shows a high degree of evolutionary conservation: five cysteines (#38, #85, #97, #132, #184 in *K. pneumoniae*) are conserved. Our goal is to define the functions of these cysteine residues and other conserved residues with regard to catalytic activity during turnover and apo MoFe protein synthesis. Data on activity and stability of these mutants will be presented.

A-12 Site-directed mutagenesis of the proposed MgATP binding region of nitrogenase iron protein from *Azotobacter chroococcum*.

L. C. Seefeldt*, R. Robson and L. E. Mortenson. Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602.

For each electron transferred from nitrogenase Fe protein to the MoFe protein, 2MgATP are hydrolyzed to 2MgADP + 2 Pi. We have undertaken site-directed mutagenesis of the iron protein from *A. chroococcum* (*Ac*II) in an attempt to identify amino acids that are involved in ATP binding and hydrolysis. We have identified a region near the N-terminus of nitrogenase Fe proteins that is a possible ATP binding site (Robson (1984) FEBS Lett., *173*, 394-398). The *Ac*II sequence $G^9K^{10}G^{11}G^{12}I^{13}G^{14}K^{15}$ has the same conserved motif (GXXXXGK) found in the mononucleotide binding loop. We have changed G11 in this region to a P. This protein (G11P) and the wild-type were expressed in *E. coli* at 2 to 3 times the levels observed in *A. vinelandii* as determined by western immunoblots. While the wild-type Fe protein expressed in *E. coli* was active in acetylene reduction activity when complemented with *Av*I, G11P showed no activity under these conditions. The wild-type and G11P proteins have been partially purified and key properties are being characterized.

A-13

A hybrid Azotobacter vinelandii - Clostridium pasteurianum Fe protein that has in vivo catalytic activity. M. R. Jacobson* and D. R. Dean. Dept. Anaerobic Microbiology, VPI&SU, Blacksburg, VA 24061, USA.

Although most heterologous Fe protein and MoFe protein complexes are catalytically active, a reconstituted mixture of C. pasteurianum Fe protein (Cp2) and A. vinelandii MoFe protein (Av1) is ineffective in substrate reduction due to the formation of a tight, inactive complex. Differences in the amino acid sequence of Cp2 when compared to Av2 have been used in attempts to explain this phenomenon and to assign a potential component protein interaction site located upon the Fe protein. Av2 and Cp2 have high levels of sequence identity throughout their primary sequences. Particularly strong sequence conservation is located in the amino-terminal region of the polypeptides corresponding to a proposed ATP-binding domain and in regions surrounding the two conserved cysteinyl residues proposed as the Fe:S cluster ligands. The most striking difference between these proteins is that Av2 is elongated by 13 residues when compared to Cp2. These 13 amino acids include five negatively charged residues and one positively charged residue. It has been suggested that these differences between Av2 and Cp2 in size and charge density could account for the formation of the tight, ineffective complex in heterologous Av1-Cp2 reconstitution experiments. We have examined this hypothesis in vivo by constructing a hybrid Av2 protein which has its carboxyl-end replaced with the corresponding Cp2 sequence. The strain which produces this hybrid Fe protein, A. vinelandii DJ319, is capable of diazotrophic growth and exhibits elevated levels of activity and accumulation for both the MoFe protein and the hybrid Fe protein in crude extracts. These results demonstrate that the tight, inactive complex formed in Av1-Cp2 heterologous reconstitution experiments cannot be accounted for only by the differences in the carboxyl-termini of the A. vinelandii and C. pasteurianum Fe proteins.

A-14

Modification of dinitrogenase reductase in the cyanobacterium *Anabaena variabilis*. A. Ernst* and P. Böger. Lehrstuhl für Physiol. und Biochem. der Pflanzen, Universität Konstanz, D-7750 Konstanz, Germany.

In the heterocystous cyanobacterium *A. variabilis* ATCC 29413 a change in nitrogenase activity and concomitant change in the electrophoretic mobility of the dinitrogenase reductase (Fe-protein) was detected with an antibody produced against this protein (1). Modification was induced under aerobic conditions either by NH_4Cl at pH 10 or by cessation of C-supply from photosynthesis, stored glycogen or exogenously added fructose. This modification was reversed under anaerobic conditions (in presence of H_2, light, and DCMU) or upon reestablishing diazotrophic growth conditions in presence of air-level oxygen (2). Under diurnal growth conditions nitrogenase exhibited lowest activity at the end of the dark period and maximum activity after 6 h of illumination. The Fe-protein became completely modified during the dark period. Demodification and activation of nitrogenase activity occurred within 1 h illumination and was followed a by further increase of activity due to nitrogenase synthesis. Addition of NH_4Cl (pH 7) represses synthesis of nitrogenase but not the activation response towards light of existing nitrogenase (3). The relevance of nitrogenase modification with respect to regulation of nitrogenase activity and maintenance of a constant C:N-ratio towards environmental changes will be discussed.

(1) S. Reich, P. Böger, 1989, FEMS Microbiol. Lett. 58:81-85
(2) A. Ernst, S. Reich, P. Böger, 1990, J. Bacteriol., in press
(3) A. Ernst, Y-D- Liu, S. Reich, P. Böger, 1990, Botanica Acta, in press

A-15 Investigation of the Paramagnetism of the VFe protein of V-Nitrogenase from *Azotobacter vinelandii*. Melinda E. Oliver[1], Roland Tittsworth[1*], Carol Blanchard[1], P. Scott Barrilleaux[1], Michael K. Johnson[2] and Brian J. Hales[1], (1) Dept. of Chemistry, Louisiana State University, Baton Rouge, LA 70803-1804, USA and (2) Dept. of Chemistry, University of Georgia, Athens, GA 30602, USA.

The VFe protein of V-nitrogenase exhibits 3 paramagnetic signals where 2 of the signals are observable in the absorption mode EPR spectrum. One of these latter signals has axial symmetry, is centered at $g < 2$ and arises from an $S = 1/2$ state most likely associated with a reduced Fe/S cluster. The redox and pH dependency of the amplitude of this signal further suggests that the metal cluster possesses a very low mid-point potential. During enzymatic turnover, the amplitude of this signal decreases, demonstrating that the metal cluster producing this signal can participate in some way in the catalytic mechanism.

A-16 Nitrogen Coordination to VFe Protein of Alternative Nitrogenase from *Azotobacter vinelandii*. Hans Thomann[1], Marcelino Bernardo[1] and Brian J. Hales[2*], (1) Corporate Research Laboratories, Exxon Research and Engineering Co., Annandale, NJ 08801, USA and (2) Dept. of Chemistry. Louisiana State University, Baton Rouge, LA 70803-1804, USA.

Previous Electron Spin Echo Envelope Modulation (ESEEM) studies of MoFe protein from *Clostridium pasteurianum* indicated that the iron-molybdenum cofactor (FeMoco) is ligated to the protein by a histidine residue. An ESEEM investigation of the $S = 3/2$ signal of V-nitrogenase shows that the cluster associated with this signal is also coordinated to the VFe protein by a deprotonated N-donor ligand whose quadrupole parameters are those expected for the imino nitrogen of an imidazole, indicating protein ligation by a histidine residue. If this signal is associated with FeVco, the similarity of the nitrogen quadrupole parameters deduced for both the MoFe and VFe proteins suggest that the imidazole imino nitrogen is coordinated to an Fe atom of the cofactor.

A-17 Metal regulation of alternative nitrogenase gene expression in Azotobacter vinelandii. R.N. Pau*, L.A. Mitchenall and F. Luque. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, Sussex, U.K.

A. vinelandii has two alternative nitrogenases, a vanadium nitrogenase, and nitrogenase-3, which apparently lacks both molybdenum (Mo) and vanadium (V). Mo plays a central role in regulating synthesis of both alternative nitrogenases, for in its presence the structural genes for V nitrogenase and nitrogenase-3 are not expressed. The structural genes for nitrogenase-3 are also not expressed in the presence of V. We describe experiments which show that repression of the structural genes for nitrogenase-3 requires the presence of the genes vnfDGK for the VFe protein of V nitrogenase. Regulation of nitrogenase-3 by V is effected by a different mechanism from the regulation of both alternative nitrogenases by Mo. Repression of the alternative nitrogenases by Mo is dependent on other genes. These and other studies on the metal regulation of the A. vinelandii nitrogenases provide elements for a model of transcriptional regulation of A. vinelandii nitrogenase structural genes by metals.

A-18 Investigation of the Nitrogenase Metal Clusters using Spin Probes. Melinda E. Oliver* and Brian J. Hales, Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803-1804, USA.

Rare-earth ions have been used in the past to relieve the microwave power saturation of paramagnetic centers and thus enhance their signals. The degree on enhancement depends on the ion's concentration, spin and relaxation time as well as the distance of closest approach of the external ion to the paramagnetic center under investigation. This means, therefore, that this enhancement can be used to probe the structure of the environment around the paramagnetic center. Because nitrogenase contains several different paramagnetic clusters, this enzyme is an ideal candidate for study by rare-earth ions. Using low-temperature EPR spectroscopy, a progressive power saturation of the nitrogenase paramagnetism was studied as a function of concentration of dysprosium ion, Dy^{3+}. By varying the environment of the enzyme, structural information is obtained.

A-19 Metal complexes as models for nitrogenase substrate-reducing sites. D.L. Hughes, D.J. Lowe, R.H. Morris, A.J.L. Pombeiro and R.L. Richards*. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

Molybdenum complexes having phosphorus or sulphur ligation are able to bind and reduce nitrogenase substrates such as N_2, H^+, RC≡CH and $H_2NC{\equiv}N$, and thus indicate likely mechanisms for nitrogenase function. This poster highlights examples of such interactions, particularly the binding of $H_2NC{\equiv}N$ at phosphorus-ligated molybdenum, and the interaction of H^+, H_2 and D_2 at sulphur-ligated molybdenum centres. The difficulty of binding and reductively cleaving organic cyanides at metal centres, and on nitrogenase, will also be discussed.

A-20 Inorganic models of *nitrogenase* attached to a polymer support. Control of redox reactions in ammonia formation. T. Adrian George* and Bharat B. Kaul, Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304, USA.

Molybdenum and tungsten complexes of molecular nitrogen (N_2) of the type **trans**-$[Mo(N_2)_2(PMePh_2)_4]$ have been investigated as "models" of nitrogenase because in reactions with acid ammonia is formed. Of equal importance is the fact that complexes containing intermediate nitrogen hydride ligands such as NNH_2 have been identified in solution and isolated and characterized. A major pathway by which nitrogen hydrides decompose is disproportionation. Recently we have attached molybdenum-N_2 complexes to a microreticular, phosphine (-$P(Ph)CH_2CH_2PPh_2$)-substituted polystyrene-2% divinylbenzene resin in order to site-isolate the metal centers and hence prevent disproportionation. Reaction of these complexes with acid proceeded to give the NNH_2 intermediate but **no ammonia** in tetrahydrofuran. In CH_2Cl_2 hydrazine was produced but **no ammonia. Ammonia is formed** when a suitable external reducing agent is added in conjunction with acid. The suitable reducing agent was a soluble molybdenum-N_2 complex that can form ammonia. Thus a polymer-supported $^{15}N_2$-labelled molybdenum complex was reacted with **trans**-$[Mo(N_2)_2(PMePh_2)_4]$, **1**, and acid. Both $^{15}NH_3$ and $^{14}NH_3$ were formed. The overall yield of ammonia per mol of **1** was 33% greater than with **1** on its own with acid. A similar reaction with a soluble N_2 complex that does not produce ammonia with acid did not produce any $^{15}NH_3$ or $^{14}NH_3$. Preparative procedures and mechanistic aspects of this work will be detailed.

A-21

Characterization of a Large-scale Fermentation of Azotobacter vinelandii.

J.-F.P. Hamel*. T. Salifoglou. R.J. Murray. A.-F. Miller. D.W. Wright. D. Hopkins. A.B. Hickman; C.L. Cooney and W.H. Orme-Johnson. Depts. of Chemistry and Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

A fermentation on the 1000-L scale, initially implemented to support research on the nitrogenase enzyme, has been characterized with respect to oxygen uptake and carbon dioxide evolution rates, cell yields on carbon and oxygen, growth and enzyme production kinetics. HPLC and MS were used throughout the fermentation to monitor sugar utilization and the composition of the outlet gas, respectively. Based on the data obtained, a carbon balance was carried out around the bioreactor, and the controlling mass transfer rate was determined. During the exponential phase, the culture growth rate peaks around 0.3 h^{-1} and the *in vivo* nitrogenase activity typically exhibits a bell-shape profile. An exponential, fed-batch strategy was implemented using a 25% solution of sugar to maintain the sugar concentration around 17g/L and to investigate the effect of high sugar concentration on nitrogenase activity. Use of a disc-stack centrifuge resulted in a reproducible high yield cell recovery. Under the operating conditions employed, greater than 10g Mo-Fe protein could be consistently recovered from a 1000-L batch.

A-22

Site-directed mutagenesis of Azotobacter vinelandii Ferredoxin I.

Siiri E. Iismaa*, Ana E. Martin, Mai-Phuong T. Nguyen, C. Dave Stout, Gerard M. Jensen, Philip J. Stephens and Barbara K. Burgess. Department of Molecular Biology & Biochemistry, University of California, Irvine, CA 92717.

We are using the 7 Fe ferredoxin from Azotobacter vinelandii (AvFdI) to study the interrelationships between protein function, protein structure and [Fe-S] cluster structure, redox properties and reactivity. AvFdI contains one [4Fe-4S] cluster coordinated by Cys residues at positions 20, 39, 42 and 45, one [3Fe-4S] cluster coordinated by Cys residues 8, 16 and 49 and two free Cys residues, 11 and 24. We have previously constructed a site-directed mutant version of AvFdI (C20AFdI) in which the Cys residue 20 has been changed to Ala. Characterization of the mutant protein by X-ray crystallographic and spectroscopic methods demonstrates that the [4Fe-4S] cluster is retained, but that the protein structure is altered to allow Cys residue 24 to serve as a cluster ligand (Martin, et al., PNAS, in press). Five additional mutants C24AFdI, C20A/C24AFdI, C11SFdI, C11VFdI and C42AFdI have now been constructed. C24AFdI and C20A/C24AFdI have been overexpressed in A. vinelandii approximately 50-fold relative to wild-type. Unlike C20AFdI, the C24AFdI protein is as stable as the wild-type protein in vivo. The C24AFdI protein has been purified in large quantities (400 mg/kg cells), diffraction-quality crystals have been obtained and its characterization will be presented. Purification of C20A/C24AFdI is in progress as is overexpression of C11SFdI, C11VFdI and C42AFdI. The functionality and redox properties of these mutant AvFdIs will be discussed.

A-23

Properties of three ferredoxins from the phototrophic bacterium Rhodobacter capsulatus: biochemical and genetic studies of their role in N_2 fixation. Y. Jouanneau, E. Schatt, C. Duport, C. Meyer and P.M. Vignais*. Biochimie Microbienne/LBIO/DRF, (CNRS UA 1130) CENG, 85X, 38041 Grenoble cedex, France.

Three ferredoxins have been isolated and purified from R. capsulatus grown under nif derepressing conditions. FdI, a 2|4Fe-4S| ferredoxin of 64 amino acids was previously shown to catalyze in vitro electron transfer to nitrogenase (1). Its structural gene, fdxN, was recently cloned, sequenced and mapped inside a main nif gene cluster of the R. capsulatus chromosome; as with nif genes, fdxN transcription was repressed by excess ammonium ions (2). FdII was about 5 times less abundant than FdI in cells grown under conditions of N-limitation but its synthesis was not repressed in cells supplied with excess ammonium. FdII exhibited physical and chemical properties similar to the well-characterized 7Fe ferredoxin I from Azotobacter vinelandii. The gene coding for FdII was cloned and sequenced. The FdII polypeptide contained 112 amino acids and exhibited 65% sequence homology with A. vinelandii FdI. FdII appeared as effective an electron carrier to nitrogenase as FdI in in vitro tests. A third ferredoxin, FdIII, was purified from N-limited cells. FdIII consisted of a polypeptide of 100 residues including 9 Cys, and contained two |4Fe-4S| clusters. FdIII had no detectable activity as an electron carrier to nitrogenase. Its structural gene was localized in a nif operon including nifE, nifN, nifX and nifQ. A role of FdIII in the processing of nitrogenase component I and/or FeMoCo is therefore anticipated.

1. Hallenbeck PC, Jouanneau Y, Vignais PM (1982) B. B. A. 681: 168-176
2. Schatt E, Jouanneau Y, Vignais PM (1989) J. Bacteriol. 171: 6218-6226

A-24

The Nitrogenase of Azorhizobium caulinodans: A Comparative Study.

J. Bergström*. Dept. of Biochemistry and Biophysics, Chalmers University of Technology and Göteborg University, Göteborg, Sweden.

The nitrogenase of Azorhizobium caulinodans has been studied in some details. A comparison with other nitrogenases has been done regarding spectral properties, reduction potentials, electron transfer rates, activity and "switch-off". The results of this investigation will be presented on the poster.

SESSION B

Plant-microbe interaction
B-01 - B-39

B-01

Effects of alfalfa *nod*-gene-inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. U.A. Hartwig, C.A. Maxwell, C.M Joseph* and D.A. Phillips, Dept. of Agronomy & Range Science, University of California, Davis, CA 95616 USA

Transcription of the nodulation genes *nodABC* in *Rhizobium meliloti* requires a plant flavonoid signal and *nodD*, a family of bacterial regulatory genes (*nodD1*, *nodD2*, and *nodD3*). Results from this study show that all previously identified *nod*-gene inducers released by alfalfa seeds and roots induced *nodABC-lacZ* transcription in *R. meliloti* containing extra copies of *nodD1*, but only 4,4'-dihydroxy-2'-methoxychalcone gave high levels of induction with extra copies of *nodD2*. Mixtures of the methoxychalcone and luteolin at concentrations that do not saturate *nodABC-lacZ* transcription show a positive synergism with extra NodD1 protein. The fact that higher concentrations of luteolin (25-75 n*M*) inhibit the methoxychalcone-induced *nodABC-lacZ* transcription with extra copies of *nodD2* indicates that these molecules probably compete for binding to the NodD2 protein.

B-02

Release and modification of flavonoid nodulation factors by alfalfa seeds. U.A. Hartwig* and D.A. Phillips, Dept. of Agronomy & Range Science, University of California, Davis, CA 95616 USA

Alfalfa (*Medicago sativa* L. cv Moapa 69) seeds release much larger quantities of *nod*-gene-inducing flavonoids than roots of young seedlings. The seeds release both active *nod*-gene inducers, such as luteolin and chrysoeriol (3'-methoxyluteolin), and inactive derivatives, such as luteolin-7-O-glucoside, 5-methoxyluteolin, and 3',5-dimethoxyluteolin. Although chrysoeriol is three to four times more active than luteolin at inducing *nod* genes in *Rhizobium meliloti*, luteolin probably is the more significant compound in the rhizosphere because large quantities of it are potentially available as the glucoside and 5-methoxy derivatives, while only a small amount of 3',5-dimethoxyluteolin is present as a potential source of chrysoeriol. Sterile alfalfa seeds release proteins during the first 4 h of germination, and luteolin-7-O-glucoside present in those exudates is converted to luteolin during the next 24 h. *R. meliloti* cells also can convert luteolin-7-O-glucoside to luteolin. Data indicating the significance of these and other flavonoid nodulation factors will be presented.

B-03

The effect of a seed color mutation on *nod*-gene-inducing flavonoids and nodulation in *Phaseolus vulgaris* L. M. Hungria*, C. M. Joseph and D. A. Phillips, Dept. of Agronomy & Range Science, University of California, Davis, CA 95616 USA

Phaseolus vulgaris line PI165426 WS is a white-seeded mutant of the black-seeded bean line PI165426 CS. Black-seeded beans synthesize high levels of anthocyanin, an end product of flavonoid metabolism. During early growth of the bean lines inoculated with *Rhizobium leguminosarum* bv. *phaseoli* CIAT 899, there are no differences in shoot or root dry weight, length of primary or lateral roots, or number of lateral roots. There are, however, nearly twice as many root nodules on the CS line as on the WS mutant 14 days after planting. The purpose of our studies is to test whether differences in nodulation patterns between these two lines are related to changes in *nod*-gene-inducing flavonoids released from seeds or roots. Using *R.l.* bv. *phaseoli* containing *nod-lacZ* fusions, which were generously supplied by cooperating microbial geneticists, we find that total *nod*-gene-inducing activities of both seed and root exudates from the black-seeded line CS are up to 10-fold higher than exudates from the mutant WS line. The active flavonoids released from these two plant lines are being identified and quantified.

B-04

Control of nodulation and other rhizosphere events by flavonoids released from alfalfa. D.A. Phillips*, U.A. Hartwig, C.A. Maxwell, C.M. Joseph, M. Hungria, and S.M. Tsai. Dept. of Agronomy & Range Science, University of California, Davis, CA 95616 USA

Alfalfa (*Medicago sativa* L. cv Moapa 69) seeds and roots release significant quantities of flavonoids during the first 72 h of germination. At least eight of those compounds, which were identified by HPLC retention times and spectroscopic techniques (UV-visible, NMR, MS), influence rhizosphere events. Structurally different flavonoids exuded by seeds and roots create two separate environments around the seed and root where they exert dissimilar effects on *Rhizobium meliloti* and other soil microbes. The identities of the compounds, their differential effects on various NodD proteins, and how they promote root nodulation will be described.

B-05

Enhanced nitrogen fixation by genetically improved alfalfa grown under field conditions. D. A. Phillips* and L. R. Teuber, Dept. of Agronomy & Range Science, University of California, Davis, CA 95616 USA

M69-33, a population of alfalfa that was selected from Moapa 69 for increased N_2 fixation capacity under controlled conditions, has been tested in the field for three years at two locations and for two years at two other sites. Although the tests are not yet concluded, initial results indicate that Moapa 69 and M69-33 produce equivalent forage yields, but the improved M69-33 population has a significantly higher forage N concentration. Estimates of forage N derived from N_2 were made at one site with the 15N-dilution and N-difference methods using an ineffectively nodulated alfalfa population, MN In-Sar, as the baseline control. Analyses from the 1988 growing season show that extra forage N in the M69-33 population was derived from N_2. Data from the 1989 field season are being examined.

B-06

The effect of salt stress on nodulation gene expression in Rhizobium meliloti and the release of inducing compounds from alfalfa seedlings. G. BENET, M. KASTELEIN and Y. KAPULNIK*, ARO, The Volcani Center P.O. Box 6, Bet Dagan, 50250 Israel.

The effect of salinity on Medicago sativa L. and R. meliloti symbiosis was assessed at the early stages of the symbiosis establishment. Transcription of nodABC genes was assayed by ß-gal activity transcribed from the nodC-lacZ fusion on plasmid pRmM57 in R. meliloti strain 1021. Low salt levels (about 100 mM NaCl), known to inhibited alfalfa nodulation, did not affect nodC-lacZ transcription. Transcription of nod genes at different stages of bacterial growth was not affected by salinity levels of up to 250 mM. Fifty percent inhibition in nodC-lacZ expression was obtained at salt concentration of 300 mM NaCl in the assay medium. The nod-inducing capacity of compounds released from imbibed alfalfa seeds was determined for 4 different cultivars under optimal and salt conditions. Although differences between cultivars were found, in each cultivar the unfractionated seed eluate released with or without 250 mM NaCl induced the transcription of nod genes to the same extent. Production and release of nodC-lacZ inducers from alfalfa roots during the first week of development was affected by different NaCl levels. In conclusion, The absorption of Rhizobium nodulation gene inducing compounds was not affected by salt, but may play a role in their production and release from alfalfa root.

B-07

An extracellular, biovar-specific signal of *Rhizobium leguminosarum* biovar *viciae* induces increased *nod* gene-inducing activity in root exudate of *Vicia sativa* ssp. *nigra*.

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Signals of *Rhizobium* induce nodulation-related root phenotypes on several leguminous plants (1,2,3). Here we report the induction of a new phenotype of *V. sativa* ssp. *nigra*, by a *Rhizobium* signal, namely the induction of synthesis and/or secretion of *nod* gene inducing activity. This additional *nod* gene inducing activity is present in root exudate and is induced by a soluble, biovar specific, extracellular signal of low molecular weight, which requires the *nod* genes *LEFDABC(IJ)* of *Rhizobium leguminosarum* bv. *viciae*.

1) Van Brussel, A.A.N. et al. (1986). J. Bacteriol. 165: 517-522.
2) Zaat, S.A.J. et al. (1987). J. Bacteriol. 169: 3388-3391.
3) Faucher, C. et al. (1988). J. Bacteriol. 170: 5489-5499.

B-08

Inoculation of *Vicia sativa* with *Rhizobium leguminosarum* biovar *viciae* increases the number of *nod* gene inducing flavonoids secreted by the roots of the host plant.

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Recently it has been shown that sterile root exudate of *Vicia sativa* contains flavonoids which activate transcription of the inducible nodulation genes of *Rhizobium leguminosarum* bv. *viciae*. The present study showed that inoculation of *Vicia sativa* with nodulating *Rhizobium leguminosarum* strains increases the *nod* gene inducing activity of the root exudate. HPLC analysis revealed 8 major inducing compounds in root exudate of inoculated plants. Sterile root exudate and exudate of plants inoculated with a Sym plasmid-cured *Rhizobium* strain contained only two *nod* gene inducers. NMR analysis showed that all 8 inducers are flavonoids which are structurally closely related.

1. S.A.J.Zaat et al. Analysis of the major inducers of the *Rhizobium nodA* promotor from *Vicia sativa* root exudate and their activity with different *nodD* genes. Plant Molecular Biology 13: 175-188, 1989

B-09 Purification of *Bradyrhizobium nod*-gene inducers from peanut.

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Three major and two minor *nod*-gene inducers were purified from the seeds of peanut. Besides inducers, inhibiting compounds were also found to be present in peanut seeds. The inducing and inhibiting activities, respectively, were tested using the *NodY-LacZ* fusion, originally isolated from *B. japonicum* USDA110, in *Bradyrhizobium* sp. NC92. Two of the inducers appear to be predominantly located in the cotyledons, and two are located in the coat of the seed. The latter ones copurify with the pigments which give the peanut seed coat its red color. Remarkably, the isolated peanut inducers show no inducing activity when tested on the *NodY-LacZ* fusion in *B. japonicum* USDA110. However, the active inducers of strain USDA110, genistein and daidzein, were found to be very active on the peanut *Bradyrhizobium* strain, even at lower concentrations than found for USDA110.

B-10 *Bradyrhizobium japonicum nodD* can specificially be induced by soybean seed extract compounds which do not induce the *nodYABC* genes.

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Besides genistein and daidzein, which are active inducers of both *nodD* and *nodYABC* in *B. japonicum*, soybean seed extract was also found to contain a number of compounds which enhance the induction of *nodYABC* genes in the presence of suboptimal genistein concentrations. This synergism was studied in further detail, and it appeared that certain compounds present in seed exudate do not induce *nodYABC*, but do induce *nodD* as determined by *nodD-* and *nodY-lacZ* fusions. Therefore, our current hypothesis is that the observed synergism is caused by a specific induction of *nodD*, leading to a higher amount of nodD protein in the cell.

NodD-inducers from soybean SE have been purified by HPLC and are now being characterized chemically. Both root exudate and seed exudate appear to contain these compounds.

B-11

Chemotaxis of *Bradyrhizobium japonicum* towards phenolic acids

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The chemotaxis of *Bradyrhizobium japonicum* 110*spc*4 was investigated with the help of a capillary assay. Succinate grown cells did not exhibit chemotaxis towards the isoflavonoids genistein, coumestrol, and daidzein present in soybean roots but were strongly attracted by another group of phenolic compounds, the phenolic acids, which are closely linked to the isoflavonoid biosynthesis. In particular cinnamic acid, coumaric acid, caffeic acid, ferulic acid, and sinapic acid were strong attractants, while derivates of these compounds (chlorogenic acid and coniferyl alcohol) did not attract *Bradyrhizobium japonicum*. Threshold values and peak concentrations for the chemotaxis towards the phenolic acids are given. The importance of the results for the *Rhizobium*-legume symbiosis is discussed.

B-12

ISOFLAVONES AND THEIR GLUCOSIDES FROM HOST PLANT THAT INDUCE NODULATION GENE EXPRESSION IN RHIZOBIA ARE ALSO POTENT AND SPECIFIC CHEMOATTRACTANTS.

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Daidzein (D) and genistein (G) and other isoflavones are known potent and specific inducers of common nodABC gene expression in Bradyrhizobium japonicum (Kosslak et al., Proc. Natl. Acad. Sci., USA, 84:7428-32, 1987). Our capillary assay showed 10^{-7} M (D) and 10^{-8} M (G) also to be potent chemoattractants for B. japonicum (about 10-fold lower than that required for gene induction). (D) showed greater response than (G). On testing the chemotaxis of Tn5 mutants of B. japonicum viz. AN122 (nodC$^-$), AN240 (nodD$^-$) and AN318 (nodIJ$^-$), we found that Tn5 mutations in nodD and nodIJ almost abolished chemotaxis to (D). However, it was not clear why nodC$^-$ did not affect chemotaxis to (D). HPLC analysis of soybean extract of seed/root showed >75% of (D) and (G) existed in glycosylated forms. Their monoglucosides showed lesser chemotaxis than the free forms. Interestingly, the diglucoside of (D) gave maximum chemotaxis (R=9.71) at 10^{-6} M and (G) diglucoside peaked at 10^{-8}M (R=7.42), indicating that rhizobia are first attracted by (G) diglucoside towards the host with high chemotactic ratio (R=6.75) at very low concentrations (10^{-9} M). Induction of nodABC genes and specific chemotaxis elicited by isoflavones, thus, appear directly related and require functional nodD gene expression. However, these two responses differ regarding the effect of the diconjugates which are less active as inducers of nod genes but are strong chemoattractants.

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B-13

Regulation of R. meliloti nodC expression by seed exudates of isogenic lines of chickpea (Cicer arietinum). Jacqueline Heard* and Thomas M. Davis. Dept. of Plant Biology, the University of New Hampshire, Durham, NH 03824, USA.

Induction of the R. meliloti strain JM57 nodC/lacZ gene by seed exudates from four nodulation mutant lines of chickpea, their wild type progenitor, and alfalfa has been examined using a β-galactosidase bioassay. At a low seed extract concentration, induction (an increase in β-galactosidase activity above basal level) was up to five times greater with alfalfa as compared to chickpea exudate. At a higher (3X) extract concentration, chickpea produced an inhibitory effect (enzyme activities below basal level), while alfalfa extract was still strongly inductive. No significant differences have been observed among the five chickpea lines in their ability to induce or inhibit nodC expression.

B-14

Effect of various N sources on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. Myeong-Je Cho* and James E. Harper. Dept. of Agronomy and USDA/ARS, Univ. of Illinois, Urbana, IL 61801, USA.

The isoflavonoid compounds, daidzein, genistein, and coumestrol, have previously been isolated and identified as inducers of *nod* genes of *Bradyrhizobium japonicum*. It is well known that soil N inhibits nodulation and N_2 fixation. This study evaluated the effect of various N sources on isoflavonoid concentration in soybean root extracts from selected nodulation mutants and the Williams wild type. HPLC analyses of soybean root extracts showed that all three hypernodulating mutants (NOD1-3, NOD2-4, and NOD3-7) and a nonnodulating mutant (NN5) had a markedly higher isoflavonoid concentration than did the Williams control when inoculated and grown at 0 mM N level. N application decreased the concentration of all three isoflavonoid compounds in all soybean lines. Application of urea and $(NH_4)_2SO_4$ resulted in less concentration of daidzein (55.8 to 85.5%), genistein (24.9 to 66.9%), and coumestrol (42.2 to 89.9%), relative to respective controls without N. Application of NO_3^- was more inhibitory to isoflavonoid concentration; resulting in 36.5 to 54.7% daidzein, 12.1 to 32.2% genistein, and 9.6 to 23.9% coumestrol, relative to respective controls without N. The degree of inhibition by NO_3^- was concentration-dependent. Although these results are consistent with a conclusion that differential NO_3^- inhibition of nodulation may be partially due to changes in isoflavonoid levels, the similar response noted for the NN5 line brings this conclusion into question. On the other hand, nodulation control in the NN5 mutant may be due to factors totally unrelated to isoflavonoids, leaving open the possibility that isoflavonoids play a role in NO_3^- inhibition of nodulation of lines genetically competent to nodulate.

B-15

Effect of Genistein on Cytokinin Production by Bradyrhizobium.

B. J. Taller* and W. A. Mulroy, Biology Department, Memphis State University, Memphis, TN 38152

Previously, we have shown that soybean seed extract caused a change in the cytokinin profile of Bradyrhizobium japonicum cultures. Thus, it was of interest to determine whether genistein would produce the same result. B. japonicum 110, containing a nodY::lacZ fusion, was grown in 3-liter fermentor jars in RDY medium +/- 2 uM genistein. Aliquots were removed from the cultures every 12 h and were assayed for galactosidase activity and cytokinins. Carbohydrate production differed in induced cultures, as there was considerably less pelletable polysaccharide than in the controls. Cytokinin content was monitored by immunoassay with anti-trans-zeatin riboside monoclonal antibodies. The cytokinin content of both cultures was similar until 48 h, when immunoreactive cytokinins decreased nearly 25% in the induced culture relative to the control. Also indicative of a shift in cytokinin species was a bioassay of Sephadex LH-20 fractionated culture medium which showed different cytokinin profiles in the two cultures after 84 h. A cytokinin-active compound, not seen in the dozens of rhizobial cultures previously tested, was found in both cultures and is being analyzed. Cytokinin production by the parent strain, B. japonicum 110, is also being examined.

B-16

Glyceollin resistance of *Bradyrhizobium japonicum* is specifically induced by isoflavonoids from *Glycine max*.

Martin Parniske*, Bettina Ahlborn and Dietrich Werner. Philipps-University of Marburg, Dept. of Biology, 3550 Marburg, FRG.

The antibacterial effect of the soybean phytoalexin glyceollin was analysed using a liquid microculture technique. Growth of *B. japonicum* exhibited a concentration dependent lag-phase. This lag-phase was followed by a logarithmic growth, indicating that the cells had overcome the inhibiting effect of glyceollin. *B. japonicum* became resistant during incubation in glyceollin containing medium. Glyceollin resistance of *B. japonicum* is not due to degradation. The isoflavonoids coumestrol, daidzein or genistein, previously detected in the rhizosphere of *G. max* are able to induce glyceollin resistance in *B. japonicum*. The flavones apigenin, luteolin or the flavanone naringenin had no such effect. Glyceollin resistance is also inducible by isoflavonoids in a *nodD*$_1$/*D*$_2$ mutant, indicating another recognition site for these signal substances besides the known *nodD* gene products.

B-17

Flavone dependence for *Rhizobium* elicitation of peroxidases from the white clover root surface. J.L. Salzwedel* and F.B. Dazzo, Dept of Microbiology, Michigan State University, East Lansing, MI 48824,USA.

Inoculation of white clover seedlings with wild type *Rhizobium leguminosarum* bv. *trifolii* or *R. leguminosarum* bv. *viciae* increases the specific activity of salt-elutable root and root hair peroxidases. The greatest specific activity is elicited by bv. *viciae* and the activity can be localized to the sites of incipient penetration at root hair tips. Similarly, pea roots inoculated with bv. *trifolii* show the greatest peroxidase specific activity. As with pathogens, the incompatible rhizobia elicit peroxidase activity in clover root hairs more rapidly (12 hrs post-inoculation) than do compatible rhizobia (24 hrs post-inoculation). Cell-free washings of plate-grown, flavone-induced rhizobia will elicit the biovar-specific pattern of peroxidase activity in white clover roots. Plate washings from uninduced bacteria do not elicit this activity. We propose that a flavone-dependent, excreted bacterial factor contributes to host specificity by affecting a peroxidase-mediated root hair cell wall modification. Current studies include analysis of peroxidase isozymes elicited by mutant derivatives containing Tn5 insertions in pSym *nod* genes (from B. Rolfe, ANU).

B-18

Rhizosphere Interaction between Rhizobium and *Glomus macrocarpum* Tul. and Tul. and a possible influence on nodulation of Alfalfa and Trifolium. N.A.PATTERSON,* S. WININGER and Y. KAPULNIK, ARO, Volcani Center, Box 6, Bet Dagan, 50250 Israel.

The beneficial effect of legume plants inoculated with vesicular-arbusculsr mycorrhiza (VAM) fungus and Rhizobium spp. is a well recognized aspect of plant microbial interaction. Recently, we reported on a specific distribution of Rhizobium nodules constructed along 18 cm of mycorrhitic *Medicago sativa* L. and *Trifolium alexandrinum* L. root system as compared to a non-mycorrhitic plant. This distribution, however, did not relate to enhancement of phosphorus uptake as a result of mycorrhizal establishment. Using the MPN technique and antibiotic resistant mutants, it was established that the number of Rhizobium cells in rhizosphere of mycorrhitic *M. sativa* L. did not change significantly along the aerial part of an 18 cm root system compared to an identical rhizosphere region of non mycorrhitic plant. Extraction of flavonoids fractions of both treatments and subsequent bio-assay showed significant influence on Rhizobium nodulation gene transcription. The expression of nodulation genes, required for Rhizobium nodule initiation was dramatically reduced when exposed to mycorrhitic flavonoid root extraction.

B-19

Had and Noi Signals from Rhizobium trifolii. R. Hollingsworth[Φ†], A. Squartini[†], S. Philip-Hollingsworth[†], and F. Dazzo*[†]. Depts. of Biochemistry[Φ] and Microbiology[†], Michigan State University, East Lansing, MI 48824, USA.

Extracellular metabolites were isolated from the culture supernatant of Rhizobium trifolii ANU843 and bioassayed for their ability to elicit morphological changes on white clover roots under axenic conditions. Supplementing the culture medium with apigenin significantly increased the accumulation of several metabolites. Among these, bacterial factors BF-3 and BF-4 were studied further. BF-3 can elicit exaggerated Had responses on root hairs and increase the number of cortical foci with cell divisions leading to nodule-like primordia. BF-4 alone is a weaker Had and Noi signal, but can enhance these root responses synergistically with BF-3. Pretreatment of white clover roots with BF-3 before cell inoculation significantly increased the number of infected root hairs and nodules formed by wild type ANU843, and enabled a nodA::Tn5 mutant derivative (ANU252 from B. Rolfe) to produce hyaline "bright spots" on deformed root hairs as an early step of infection thread formation. Thus, BF-3 and BF-4 are likely to be "nod signals" from R. trifolii.

B-20

Antigenic changes in lipopolysaccharide (LPS I) and an outer membrane protein of Rhizobium leguminosarum bv. viciae 248 during the differentiation of bacteria to bacteroids in nodules of Vicia sativa. L. Goosen-de Roo* and R.A. de Maagd. Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands.

During the differentiation from bacteria to bacteroids the cell surface of Rhizobium undergoes a number of dramatic changes. Monoclonal antibodies were used for immunogold localization of LPS I epitopes and of an outer membrane protein (OMP) antigen of Rhizobium leguminosarum bv. viciae 248 in nodules of Vicia sativa to determine the kinetics of disappearance. LPS I epitopes were located on the outer membrane of bacteria in infection threads in the nodule after immunogold labeling with three monoclonal antibodies. These epitopes were not detectable on rhizobia released from the infection thread. The OMP antigen was also located on bacteria in infection threads, was present in slightly lower amounts on rhizobia just after release from the infection thread, and in much lower amount in mature bacteroids. These results show that the OMP antigen disappears gradually during the differentiation of the bacteroids whereas the LPS I epitopes seem to disappear rather abruptly after the release from the infection thread. The results are consistent with the notion that upon release of the bacteria in the infection thread the synthesis of the OMP antigen stops whereas the LPS I epitopes are actively degraded.

B-21

Isolation and analysis of the lipopolysaccharides from symbiotic mutants of *B. japonicum*. R.W. Carlson*, B. Lakshmi, U.R. Bhat and G. Stacey. Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602 and Dept. of Microbiology, University of Tennessee, Knoxville, TN 37996.

The lipopolysaccharides (LPSs) from symbiotic mutants of *B. japonicum* 61A101C, J314 and ML142 were partially characterized by PAGE and glycosyl composition analysis. The mutants form empty nodules that have central rather than the normal peripheral vascular tissue. The LPSs are extracted into the phenol layer during phenol/water extraction and further purified by treatment with proteinase K, RNase, DNase and gel-filtration chromatography. PAGE was performed in buffer containing deoxycholate (DOC) and glycosyl components were determined by preparing alditol acetates followed by GC/MS analysis. PAGE analysis shows that the mutants lack the higher molecular weight form of LPS, LPS I. The parent LPS is composed of 2,3-di-O-methylfucose, fucose, rhamnose, 4-O-methylmannose, mannose, galactose, glucose, quinovosamine and fucosamine. The major sugars in the mutant are 4-O-methylmannose, mannose and glucose. Major sugars in the parent LPS, e.g. 2,3-di-O-methylfucose and fucose, are missing in the mutant LPSs. Fatty acids identified in both parent and mutant LPSs are: C12:OH, C12, C14:OH, C14, C16, C18:1, C18, C20:1 and C24. The long-chain fatty acid, C28:27-OH is also detected.

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B-22

Immunological dissection of the plant-microbial interface in pea nodule symbiosomes. N.J. Brewin*, S. Perotto, E.A. Wood, K.A. VandenBosch and E.L. Kannenberg. IPSR John Innes Institute, Colney Lane, Norwich NR4 7UH, UK.

The object of this study was to investigate the role of surface interactions between plant-derived peribacteroid membranes and endosymbiotic Rhizobium bacteroids in pea nodules.

Using rat monoclonal antibodies, we have identified some periodate-sensitive (carbohydrate) components which probably comprise a plant glycocalyx located on the luminal face of the peri-bacteroid membrane. The carbohydrate epitopes were attached to macromolecules that were either protease-sensitive (glycoproteins) or extractable in organic solvents (glycolipids).

Bacterial lipopolysaccharide antigens were also identified using mono-clonal antibodies. Some epitopes were expressed constitutively and others were developmentally regulated within nodules. Low oxygen or low pH was shown to influence the expression of these epitopes. Rhizobium mutants that lacked particular combinations of lipopolysaccharide epitopes were unable to fix nitrogen in pea nodules.

B-23

Immunolabeling suggests uptake of a plant glycoprotein by *Rhizobium leguminosarum*. KA VandenBosch*[1], DA Dreyer[1] and NJ Brewin[2]. [1]Dept. of Biology, Texas A & M University, College Station, TX, 77843-3258, USA, and [2]John Innes Institute, Colney Ln, Norwich, NR4 7UH, UK.

In an ongoing characterization of the peribacteroid membrane of nodules of *Pisum sativum*, we have isolated a monoclonal antibody that reacted with a distinctive set of peribacteroid proteins. The epitope was a periodate-sensitive carbohydrate moiety borne on numerous glycoproteins. The antibody recognized several nodule-specific proteins in the molecular weight range 25-35 kDa. A 50 kDa glycoprotein was also detected in isolated bacteroids. This component was also found in plant cell fractions, but was absent from cultured rhizobia. Immunocytochemistry demonstrated three prominent locations of the epitope within infected cells: Golgi bodies, the peribacteroid space, and an accumulation between the bacteroids' inner and outer membranes. The latter feature represents a novel structure not previously described. The observations indicate that the labeled glycoproteins are glycosylated in the plant cell's Golgi bodies, and then transported to the peribacteroid space. The lack of glycoproteins in gram negative bacteria suggests that the 50 kDa glycoprotein is taken up from the peribacteroid space into the bacterial periplasm. Efforts to characterize and to isolate this novel glycoprotein continue.

B-24

Mutations that Alter O-antigen Structure of *R. trifolii* ANU843. K. Chapman*+, S. Pankratz+, S. Phillips-Hollingsworth+, F. Dazzo+, and S. Wright@
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Monoclonal antibodies were raised against *R. trifolii* ANU843 cells grown in the presence of the flavone apigenin. The antibodies were used to screen kan^r Tn5 mutagenized cells. Mutants lacking one or more cell surface epitopes were found at a frequency of 1 - 2%. These mutants were grouped into classes according to their binding to various monoclonal antibodies.

Rough mutants, which lack all known O-antigen epitopes, are $nod^+ fix^-$ on all clover varieties tested. Examination of clover root nodules formed by rough mutant bacteria show that some bacteria are released from the infection threads but that these usually fail to undergo normal bacteroid development (bad^-). Rough mutants will swell and assume a bacteroid shape *in vitro* when grown in liquid culture in the presence of added succinate. Some individual Red Clover plants will form a partially effective symbiosis with rough mutant bacteria.

Some mutations eliminate some but not all O-antigen epitopes or affect the ratio of complete vs. rough type LPS. These mutants produce chemically distinct LPSs but show no change in nodulation kinetics or nitrogen fixation on Dutch White Clover.

B-25

Possible involvement of nodI in down-regulation of 3-hydroxybutyrate substitution in the CPS of Rhizobium trifolii ANU843. S.Philip-Hollingsworth*, L. Cargill, and F.B. Dazzo, Dept. of Microbiology, Michigan State University, East Lansing, MI 48824, USA.

^{1}H-NMR analyses were performed on enzymatically cleaved oligosaccharides from the acidic CPS of wild type R. trifolii ANU843 and its pSym mutant derivatives (from B. Rolfe) grown with and without 7,4'-dihydroxyflavone (DHF). The oligosaccharides from the nodI mutant had more 3-hydroxybutyrate (3-HB) substitution than did wild type. The oligomers from all other mutants containing single copies of pSym nod genes were similar to wild type. However, recombinant strains of ANU845pSym containing multiple copies of certain pSym nod genes showed pronounced differences in the level of 3-HB substitution. R. trifolii strains ANU845pRt308 (containing nodABCIJ, nodD, and nodFERL), ANU845pRt032Δ910 (containing nodABCIJ, and part of nodD) grown without DHF, and ANU845pRt032 (nodABCIJ, nodD, nodFERL, and nodMN) grown with DHF all had CPS's with reduced levels of 3-HB. ANU845pRt150 (containing nodABC, nodD, and nodF) and 845pRt032 grown without DHF had similar levels of 3-HB as the wild type. The data suggest that nodI down-regulates the level of 3-HB substitution in R. trifolii. The level of 3-HB substitution is one of the major differences between the acidic CPS made by wild type R. leguminosarum bv. trifolii and bv. viciae.

B-26

Stimulation of early adsorption of *Rhizobium meliloti* to alfalfa roots, and their nodulation, by previous specific interaction with root exudate protein factor or seed agglutinin. L.G. Wall, M.C. Giménez and G. Favelukes*. Quimica Biológica I, Facultad de Ciencias Exactas, UNLP, 1900-La Plata, Argentina.

Symbiont-specific interaction of *R. meliloti* with an alfalfa root exudate (RE) macromolecular factor stimulates its early adsorption to roots (L. G. W. and G. F., 7th Int. Congr. Nit. Fix., Köln). Here we report that the stimulatory activity in RE (which is trypsin-sensitive), is specifically and completely removed from solution by either late-log or stationary phase *Rme* L5-30 ($5x10^5$ cells per ml), while the ensuing stimulation of *Rhizobium* adsorption to roots requires late-log bacteria. Pretreatment of *Rme* L5-30 with RE has also stimulated early infection and nodulation of alfalfa root as shown by the number of nodules above RT mark, position of the uppermost nodule or profiles of nodulation frequency. Similar results of stimulation of adsorption and early nodulation were obtained when alfalfa seed agglutinin replaced the RE. A root-secreted protein -probably related to alfalfa seed agglutinin- is thus involved in an early *Rme*-alfalfa recognition event, which leads to symbiotic association and nodulation.

B-27 Rhizobium meliloti LPS saccharide moiety involved in early symbiotic recognition by alfalfa.

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Crude preparations of LPS from Rhizobium meliloti (Rme), but not from heterologous rhizobia, have been previously shown to inhibit specific adsorption of Rme cells to alfalfa roots (A.L. and G.F., 7th Int.Congr.Nit.Fix., Köln). Chromatography of crude LPS on Sepharose CL-4B-EDTA-TEA has now separated three components of which only the mid-one, eluting as a partially included band (Kd=0.47) and having properties expected of Rme LPS proper (a high KDO:hexose content ratio, 0.37 (w/w), and a marked hydrophobicity as detected with the probe merocyanin 540, suggesting the presence of a Lipid A moiety) was inhibitory in the Rme specific adsorption assay. After mild acid hydrolysis of crude LPS which eliminated the Lipid A moiety, the inhibitory activity was preserved and even increased in the remaining soluble product(s), suggesting an active role for the original saccharide regions of LPS. Extensive cross-immunoreactivity among LPS of different Rme strains (but excluding heterologous LPS) gives independent evidence of the ability of LPS as a common marker of the Rme cross-inoculation group. These studies support the hypothesis that Rme LPS saccharide chains carry determinants for early symbiotic recognition by alfalfa, expressed during specific root adsorption.

B-28 Lipopolysaccharide epitopes missing from bean bacteroids.

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Previous work has indicated that *Rhizobium leguminosarum* must carry abundant lipopolysaccharide (LPS) O-antigen to elicit completely developed and infected nodules. A possible reason for this requirement is that the O-antigen undergoes modifications during legume infection that are required for symbiotic function. Recently, Brewin and colleagues (J. Bacteriol. 171: 4537-4555) demonstrated that pea bacteroids contained an LPS epitope that is not found in the same strain in the free-living state. Recent studies in this laboratory indicate that bean microsymbiont *R. leguminosarum* CFN42 undergoes a similar change. Four monoclonal antibodies prepared by immunization with the wild type or a mutant derivative cultured *ex planta* were obtained from N.J. Brewin. These antibodies were shown to recognize different epitopes within the LPS molecules that carry O-antigen. Two epitopes were missing from the LPS of bacteroids. The bacteroids also contained a minor LPS species that was not related at all antigenically to the LPS of bacteria cultured free of the plant. The LPS of certain Fix+ LPS-altered mutant constructs also changed during symbiosis, but in a manner somewhat different from that of the wild type. The epitope changes in the wild-type bacteroids could be mimicked by culture *ex planta* at pH below 5.0. Mere incubation at this pH in the absence of growth did not result in these changes. Present studies are directed at the chemical and genetic bases of these changes and their importance in the symbiosis.

B-29

Characterization of exopolysaccharide-deficient mutants of Rhizobium leguminosarum bv. phaseoli with altered nodulation competitiveness. R.S. Araujo* and J. Handelsman. Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

Rhizobium leguminosarum bv. phaseoli (Rlp) strains KIM5s, CIAT899, TAL182v, and CE3 were mutagenized with transposon Tn5 and the mutants were screened for non-mucoid colony morphology on high carbon, solid medium. A total of 70 independent mutants derived from the four parent strains was obtained. The non-mucoid phenotype correlated with lack of exopolysaccharide (EPS) production in broth culture. All EPS-deficient mutants induced normal, nitrogen-fixing nodules on beans (Phaseolus vulgaris L.). The nodulation competitiveness of 10 EPS-deficient mutants of strain CE3, four of strain KIM5s, and four of strain CIAT899 was determined in experiments in which bean seeds were inoculated with each mutant paired with its parent. Nodulation competitiveness was assessed by determining the proportional representation of each strain in the nodules relative to its proportional representation in the inoculum. EPS-deficient mutants of strains KIM5s and CIAT899 were consistently less competitive than their parents, whereas mutants of strain CE3 were either reduced, unaltered, or increased in nodulation competitiveness relative to the parent. One EPS-deficient mutant of strain CE3 had been complemented to restore EPS production with a cosmid containing the appropriate insert of parental DNA and the restored strain was as competitive as the parent strain. These results suggest that mutations that affect EPS production may directly or indirectly affect nodulation competitiveness in Rlp. A further genetic analysis will enable us to determine the genetic and physiological relationship between EPS production and nodulation competitiveness in Rlp.

B-30

Induction of chlorosis in bean plants (Phaseolus vulgaris) by culture filtrates of exopolysaccharide-deficient mutants of Rhizobium strains CIAT899 and TAL182v. K.P. O'Connell*, R.S. Araujo and J. Handelsman, Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

Exopolysaccharide (EPS)-deficient mutants of Rhizobium strains CIAT899 and TAL182v were generated with Tn5 and ethyl methane sulfonate (EMS). The mutants and wild-type strains all induced pink, effective nodules. The parental strain TAL182v caused no symptoms in bean plants, and CIAT899 caused slight chlorosis on a small proportion of inoculated plants. However, all EPS-deficient mutants of both strains caused severe interveinal chlorosis in the first trifoliate leaves of bean plants. Cultures of strain CT9005, a Tn5-induced EPS-deficient mutant of CIAT899 were diluted serially and applied to bean plants. Lower dilutions produced progressively less severe chlorosis. Parental strain CIAT899 also nodulates the tree legume Leucaena leucocephala; CIAT899 did not cause chlorosis in Leucaena seedlings, whereas mutant CT9005 caused interveinal chlorosis in the leaves of L. leucocephala. Culture filtrates of TL1016, a Tn5-induced EPS-deficient mutant of TAL182v, induced chlorosis in bean plants while washed cells of TL1016 did not. Neither cultures, filtrates, nor washed cells of TAL182v induced chlorosis in bean plants. These data suggest that EPS-deficient mutants of CIAT899 and TAL182v produce a phytotoxin in broth culture. We are investigating the biochemical nature of this putative phytotoxin, which induces symptoms similar to those caused by rhizobitoxine, a phytotoxin produced by some strains of Bradyrhizobium japonicum. We are also studying the intriguing relationship between EPS deficiency and the ability to cause chlorosis.

B-31

Cloning of DNA sequences conferring enhanced competitiveness in *Rhizobium leguminosarum* bv. *phaseoli*. Gwyn A. Beattie* and Jo Handelsman. Dept. of Plant Pathology, University of Wisconsin, Madison, WI 53706, USA.

The goal of our work is to understand the basis for nodulation competitiveness in rhizobia. Previously, we identified a pair of *Rhizobium leguminosarum* bv. *phaseoli* (Rlp) strains, KIM5s and CE3, whose relative competitiveness is similar in a variety of environments. KIM5s is consistently 10 to 15 times more competitive than CE3; this consistency makes these strains ideal subjects for a genetic study of competitiveness. To identify genes that contribute to the successful competitiveness of KIM5s, libraries of the KIM5s genome were moved into CE3 and the transconjugants were screened for enhanced competitiveness. In order to use the plant as the selective agent for transconjugants with enhanced competitiveness, the CE3 transconjugants were applied to bean seeds in groups of 50 and the nodule isolates from the plants were applied to seeds for a second enrichment. The competitiveness of randomly selected nodule isolates from these plants was evaluated by competing each isolate against KIM5s. Of 2400 independent transconjugants applied to plants, three nodule isolates from the final enrichment appear to be more competitive than the CE3 parent and one transconjugant is equal in competitiveness to KIM5s. The cosmids in these three transconjugants, designated pOWR1, pOWR2, and pOWR3, contain inserts that are approximately 20.5, 19.8, and 13.1 kb, respectively. Preliminary results indicate that the cosmid pOWR1, which confers competitiveness equal to KIM5s, may also confer the ability to produce melanin on *Escherichia coli*. Since melanin production is encoded on the Sym plasmid of Rlp, this suggests that pOWR1 may contain an insert from the Sym plasmid of KIM5s. These cosmids provide the means for determining the genetic and physiological basis of nodulation competitiveness.

B-32

Competition Defective Mutants of Bradyrhizobium japonicum. Arvind A. Bhagwat* and Donald L. Keister. USDA-ARS, Nitrogen Fixation & Soybean Genetics Lab, BARC-W, Beltsville, MD 20705.

There is considerable evidence from work with mutants that rhizobial extracellular polysaccharides (EPS) are important in the infection and nodulation process. Many mutants which do not produce normal amounts of EPS or have structural alterations in EPS do not nodulate, or form nodules which are ineffective. However, the precise role(s) of EPS is not known.

We have isolated Tn5 induced mutants of B. japonicum 61A89 (= USDA 110) which are deficient in capsular EPS synthesis. Some of these mutants failed to occupy nodules on Glycine max cv Williams when co-inoculated with other strains even when the populations were 10:1 in favor of the mutant. When inoculated singly, the mutants formed effective nitrogen-fixing nodules.

All the mutants exhibited altered colony morphology and produced less mucoid colonies on minimal medium with several different carbon substrates. Urea-SDS-PAGE analysis revealed that one of the mutants (A3) had altered lipopolysaccharides. The wild-type DNA segment complimentary to the Tn5 flanking regions of mutant A3 was isolated from a gene library. A 9.6 kb EcoR1 fragment complimented the competition defect of mutant A3 but not of the other mutants. This DNA also restored normal lipopolysaccharide and the mucoid colony phenotype in mutant A3. Subcloning and site-directed Tn5 mutagenesis followed by homogenotization were performed to delimit the region complimenting the mutation causing the competition defect.

B-33 The role of plasmids in competition between strains of *Rhizobium leguminosarum*.

Michael F. Hynes*, Agriculture Canada Research Station, Lethbridge, AB, Canada T1J 4B1.

Previous work from our lab has shown that all plasmids in a given strain of *R. leguminosarum* can be cured, and that two plasmids other than the nodulation plasmid carry genes necessary for the formation of a nitrogen fixing nodule (Hynes and McGregor, Molec. Microbiol., in press). This study was carried out to determine what effect, if any, the various plasmids of *R. leguminosarum* strain VF39 had on the strain's competitive ability. The two smallest plasmids, pRleVF39a and pRleVF39b, did not influence competition. The plasmids pRleVF39c (LPS genes), pRleVF39d (*nod*, *nif*), and pRleVF39e, all of which are necessary for nitrogen fixation, were also indispensable for competition. The plasmid pRleVF39f, which is not necessary for symbiosis, proved to play an important role in competition. Strains cured of pRleVF39f were less competitive than several reference strains, whereas wild-type VF39 was more competitive. Strains cured of pRleVF39f were also unable to compete successfully with genetically marked derivatives of wild-type VF39. The effect of loss of pRleVF39f on competition was reproducible in growth pouches, in sterile sand/vermiculite mixtures, and in soil. We are now in the process of cloning the regions of pRleVF39f which are important in competition.

B-34 Specific forms of exopolysaccharides from *Rhizobium meliloti* restore symbiotic effectiveness to *R. meliloti* Exo- mutants.

L. Battisti*, C.C. Lee and J.L. Leigh, Dept. of Microbiology SC-42, University of Washington, Seattle, WA 98195 USA

Rhizobium meliloti SU47 produces both high and low molecular weight (mw) forms of an exopolysaccharide, EPSA, composed of repeating octomeric subunits of B-linked glucose, B-linked galactose, acetyl, pyruvyl and succinyl substituents (7:1:1:1:1). We have separated low mw EPSA into at least four subfractions that appear to differ in subunit multiplicities (e.g. monomer, dimer, trimer, tetramer). Tn5 mutants of SU47 which fail to produce any form of EPSA (Exo-) exhibit a delay in root hair curling and induce empty non-nitrogen fixing nodules. A bioassay was developed to determine if various forms of EPSA could correct the nodule invasion defect of Exo- mutants. Low mw EPSA partially restored nodule invasion to various Exo- mutants; heat and protease treatment had no effect on this activity. High mw EPSA, monomeric and dimeric subfractions of EPSA, and high mw acidic exopolysaccharides from *Rhizobium* sp. NGR234, *R. trifolii*, *R. leguminosarum* and *Agrobacterium tumefaciens* failed to restore nodule invasion. *R. meliloti* SU47 has the cryptic ability to produce another high mw acidic exopolysaccharide, EPSB. Mutants that produce EPSB but not EPSA invade nodules. EPSB also partially restored nodule invasion in the bioassay, confirming that the two polysaccharides are functionally interchangeable. We also examined the ability of several of the above polysaccharides to induce marked root hair curling; the most effective were low mw EPSA, and EPSB. These results demonstrate that specific forms of EPSA and EPSB play a biologically active role in the *R. meliloti*-alfalfa symbiosis.

B-35

Rhizobial polysaccharides specifically inhibit the 1,3-ß-glucan synthase of *Glycine max* and *Pisum sativum*. Bettina Ahlborn* and Dietrich Werner. Dept. of Biology, Philipps-University, Marburg, FRG.

Callose is rapidly deposited after an attack by pathogens to seal plant cell walls. It is believed that the membrane-bound 1,3-ß-glucan synthase (GS II) catalizes this reaction. However, the same enzyme is possibly also involved in cellulose synthesis. Microbial polysaccharides have frequently been suggested to play an important role in recognition processes between pathogenic and symbiotic microorganisms and their respective host plants. Different polysaccharides exhibit different effects on the GS II. Bradyrhizobial CPS strongly inhibits GS II-activity of both *Glycine max* and *Pisum sativum*. In contrast, Laminarin and Xanthan do not influence the GS II-activity at all. The specific inhibition of ß-glucan-synthesis by symbiotic organisms might be of relevance for the infection process.

B-36

Toxicity of Leucaena leucocephala seed diffusate to Rhizobium. M. Alameda and E.C. Schröder*. B N F Laboratory, Dept. of Agronomy and Soils, University of Puerto Rico, Mayagüez, PR 00709-5000, USA.

Nodulation of Leucaena leucocephala was sparse and erratic under in vitro conditions. A yellow substance diffused in the agar media (Jensen) of the low nodulation tubes. It has been reported that the seeds of some leguminous species produce toxic substances that affect the survival of rhizobia. Experiments were undertaken to determine if the seeds of L. leucocephala produced substances toxic to Rhizobium. Two treatments (with and without scarification) of autoclaved seeds were tested in Yeast Extract Mannitol Agar (YEM) and inoculated with fast growing Rhizobium (TA1 and TAL 1145). Larger inhibition zones were observed in scarified seeds (20.15 mm) than non scarified seeds (7.02 mm). Sterile cotyledons were separated from seed coats and tested in YEM. Inhibition zones around cotyledons were larger (29.61 mm) than those around seed coats (7.22 mm). Filter paper discs impregnated with sterile aqueous seed extract also showed inhibition zones (17.33 mm). Survival of Rhizobium on leucaena seeds might be reduced by the presence of this toxic compound and inoculation methods should be improved to reduce this potential problem.

B-37 Regulation of Nodule Distribution Analyzed by Delayed Supplementation of Rhizobial Purine Auxotrophs. J.D.Newman*, B.W.Schultz, K.D.Noel. Biology Dept., Marquette University, Milwaukee, WI 53233

Rhizobium leguminosarum bv. *phaseoli* purine auxotrophs elicit pseudonodules devoid of bacteria and scattered throughout the bean root system. Supplementation of the root medium with 1 mM inosine or 0.1 mM AICAR, a purine precursor, allows infection and enhances nodule development. The resulting nodules are clustered near the top of the root and are the same size as those elicited by the wild type. Delaying the addition of AICAR until various times after inoculation results in enhancement of nodulation in areas of the root developing at the time of AICAR addition, rather than areas developing at the time of inoculation. When AICAR is added 1 to 5 days after inoculation, no bumps or nodules arise on or adjacent to the tap root, in contrast to what occurs in the absence of supplementation. Only if AICAR addition is delayed more than 6 days post-inoculation do pseudonodules appear on and near the tap root, in addition to enhanced nodulation in areas of the root developing at the time of AICAR addition. These observations suggest that, to be successful, infection must occur very soon after the nodule meristem forms; rescue is not possible after even 1 day. Development of nodules having sustained infections suppresses the development of pseudonodules initiated at *earlier* times as well as suppressing nodule formation further down the root. A model expanding upon the present hypotheses of nodule development is proposed to explain these observations.

B-38 Genetic complementation of Rhizobium leguminosarum bv. phaseoli Tn5 mutants defective in nodulation and nodulation suppression. M.L.F. Caces*, F.M. Robert, D. Borthakur & B.B. Bohlool. University of Hawaii, Honolulu, HI 96822.

A plant signal induced by infecting rhizobia is postulated to regulate nodulation and inhibit subsequent nodulation. Evidence for this is seen in a split-root system where a suppression of nodulation occurs on a half root when the other half has been previously nodulated. We investigated the genetics of suppression in Phaseolus vulgaris (common bean) using Rhizobium leguminosarum bv. phaseoli mutants. TAL182, a highly competitive and effective strain, was mutagenized with Tn5 and screened for symbiotic defects. Two classes of nodulation mutants and four types of auxotrophic mutants (Ade$^-$, Leu$^-$, Phe$^-$, Ura$^-$) that are blocked at different stages of symbiotic development, were identified. When the mutants were used as primary inoculants in split-root experiments, they failed to induce nodulation suppression. Complementation of the nodulation defective mutants with a cosmid library of Rhizobium sp. (Lucaena leucocephala) TAL1145, restored the wild-type nodulation and suppression characteristics. Restriction analysis of the overlapping clones complementing the Class I and Class II nodulation mutants showed an 8.3-kb region and a 12-kb region in common, respectively.

B-39

Nodulation Blocking in *Rhizobium fredii*. Pedro A. Balatti* and Steven G. Pueppke, Dept. of Plant Pathology, Univ. of Missouri, Columbia, MO 65211.

Rhizobium fredii USDA257 induces cortical cell divisions in advanced soybean cultivars such as McCall, but nodules do not form in growth pouches. Mutant 257DH4, which was derived from USDA257 by transposon mutagenesis, forms nitrogen fixing nodules under these conditions. If USDA257 is present in inocula containing the mutant, most infections are arrested prior to organization of the nodule meristem, and nodule number is reduced by 95% or more. Culture filtrates of USDA257 do not possess nodulation blocking activity, but filtrates of cells that have been pre-exposed to exudates of McCall roots can block nodulation by about 40%. Supernodulating Williams soybean is as sensitive as McCall to nodulation blocking. We have screened additional bacterial strains for the ability to substitute for USDA257 in blocking nodulation of McCall. Blocking is correlated to the ability of a strain to induce a threshold number of cortical divisions in the plant. Blocking strains USDA257, USDA193, and USDA208 all form nodules on the primitive soybean cultivar, Peking, and they do so more rapidly than does mutant 257DH4. This suggests that nodulation by 257DH4 may be inherently inefficient and that this characteristic may contribute to its sensitivity to blocking on cultivars such as McCall.

SESSION C

Limiting factors of nitrogen fixation
C-01 - C-35

C-01 Development of a hydrogen-sensitive micro-electrode for use with legume nodules.

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Until recently it has not been possible to obtain direct measurements of the concentration of H_2 in the infected region of legume nodules and estimates based on mathematical models have been controversial. We have developed a selective micro-electrode system which measures H_2 concentrations directly. The electrode is sensitive down to levels of 1000 ppm. Studies with several legume species suggest that, under normal circumstances, H_2 concentrations would not be high enough to inhibit nitrogenase activity. However, higher concentrations do occur at elevated gas phase O_2 levels and when N_2 in the gas phase is replaced with He or Ar.

C-02 Interaction of O_2 and H_2 on the in and ex planta nitrogenase activity (C_2H_2 reduction) of Hup^+ ($hydrogenase^+$) Bradyrhizobium japonicum

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Green house and field pluriannual inoculation trials comparing the efficiency of isogenic Hup^+ and Hup^- B. japonicum show no obvious superiority of the Hup^+ bacteria although it oxidizes the H_2 produced by the nitrogenase reaction (Drevon et al., 1987, 1988). Hydrogenase activity under oxygen limited conditions is considered as energeticaly unfavourable in free living fixing bacteria (Stam et al., 1987 ; Yates et al., 1988). This prompted us to measure the effect of external kPa O_2 on the acetylene reduction activity (ARA) of bacteroid suspensions and unexcised nodulated roots of the B. japonicum PJ 17 -1 (Hup^+) - Glycine max. cv Kinsoy, in presence or absence of 20kPa H_2. In absence of H_2, the ARA of bacteroid was maximum under 1kPa O_2. In presence of H_2, the optimal pO_2 and maximum ARA were slightly higher which confirms previous observations (Ruiz-Argueso et al., 1979). But under pO_2 below 1kPa, the ARA was lower with H_2 than without H_2.
For intact plants, in environmental conditions where the inhibition of nitrogenase by C_2H_2 was not observed (Drevon et al., 1988), the optimal pO_2 in absence of H_2 was 35 kPa O_2, the corresponding ARA was 110% higher than ARA under 20kPaO_2 . In presence of H_2 those parameters were 40 kPa O_2 and 140% ; however the ARA was more limited by O_2 below 20 kPa O_2 than in absence of H_2. Those data support the concept that hydrogenase reaction is unfavourable to nitrogen fixation in the rhizobium-soybean symbiosis because of O_2 limitation of nodule metabolism and relatively poor energy yield of H_2 oxidation.

C-03 Characterization of Hydrogen-Ubiquinone Oxidoreductase Activity in H_2-oxidizing *B.japonicum* Membrane Vesicles.

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H_2 oxidation in *B.japonicum* involves a branched membrane-bound electron transport chain from H_2 to O_2. The proposed sequence involves electron flow from hydrogenase to ubiquinone, then through various cytochromes to O_2. Ubiquinone has been suggested as the immediate electron acceptor from hydrogenase, but no biochemical evidence exists for direct electron transfer. Purified hydrogenase, which catalyzed methylene blue-dependent H_2 oxidation, was unable to catalyze ubiquinone-dependent H_2 oxidation. Membrane vesicles containing the entire oxyhydrogen electron transport chain were found to catalyze ubiquinone dependent H_2 oxidation in the absence of O_2 or in the presence of NaCN. Ubiquinone-10 (the physiological quinone) and the water soluble quinones ubiquinone-0 (Q-0) and ubiquinone-1 all supported H_2 uptake. Addition of Q_0 to membranes already catalyzing the oxyhydrogen reaction resulted in increased H_2 uptake and decreased O_2 uptake. The membrane-bound H_2-ubiquinone oxidoreductase activity was further characterized by the use of inhibitors. The quinone analog HOQNO inhibited both the oxyhydrogen reaction and the H_2-Q_0 oxidoreductase activity, but not methylene blue dependent H_2 oxidation. The results presented indicate that ubiquinone cannot function as a direct electron acceptor from pure hydrogenase of *B.japonicum*, and that different sites for ubiquinone reduction and methylene blue reduction exist in the oxyhydrogen electron transport chain.

C-04 The effect of H_2 on the EAC in soybean nodules.

A.H.Moloney*, R. Guy, D.B.Layzell, Dept. of Biology, Queen's Univ., Canada.

Mathematical models of gas diffusion suggest that the H_2 concentration in the infected cells of a legume nodule may be higher than the Ki for H_2 inhibition of N_2 fixation. In vitro studies have shown that H_2 and N_2 are competitive substrates of nitrogenase. This competition stimulates H_2 production, and decreases the proportion of total electron flow which is used in N_2 reduction (i.e. decreases the electron allocation coefficient, EAC).

To determine whether H_2 inhibition of nitrogenase regulates EAC in vivo, detached soybean nodules were placed in a gas exchange system, and over a 30 minute period, nitrogenase activity was measured as H_2 evolution in the presence and absence of N_2, $^{15}N_2$ incorporation, and acetylene reduction. A preliminary study showed that 30-90 minutes following detachment nitrogenase activity was stable at c.20% of the in situ level, and that EAC was similar to that in nodules in intact plants. When the pH_2 in the gas phase around the nodules was raised from 0 to 27.5 kPa, the N_2 fixation rate declined from 59.7 to 33.9 μmol N_2 fixed/ g DW nod/hr, and EAC declined from 0.61 to 0.48. These results suggest that the in vivo Ki for H_2 inhibition of N_2 fixation is higher than that for purified enzyme. The results will be used to discuss whether or not H_2 inhibition of N_2 fixation regulates EAC in vivo.

C-05

Light-driven $^{14}CO_2$ fixation and decreased O_2 uptake, and acetylene reduction activity by free-living Rhizobium strain BTAi1. M. Hungria[1], J.M. Ellis*[1], A.R.J. Eaglesham[2], and R.W.F. Hardy[1]. [1]Boyce Thompson Institute, Ithaca, NY 14853; [2]EniChem Americas, Monmouth Junction, NJ 08852.

An aerobic Rhizobium strain BTAi1 is an aerobe, isolated from sand, that nodulates both stems and roots of Aeschynomene, a tropical genus that usually grows in water-logged conditions. When exposed to light, this strain produces pink colonies, contains bacteriochlorophyll a, increases CO_2 fixation, and decreases O2 uptake—suggesting that it is photosynthetic. $^{14}CO_2$ uptake by BTAi1 was detected after 1 min of incubation in all growth stages, and the reaction was linear for 60 min. The addition of inhibitors of photosystem II, 1,10 phenanthroline, dichlorophenyldimethyl-urea and the uncoupler NH_4Cl immediately suppressed both light-driven $^{14}CO_2$ uptake and light-decreased O_2 uptake. Although BTAi1 showed photosynthetic properties, the strain could not grow in medium without C, and it was unable to grow chemoautotrophically in an atmosphere enriched with H_2 and CO_2. In microaerophilic conditions and media deficient in N, free-living BTAi1 expresses both nitrogenase and CO_2 uptake activities. Higher rates of CO_2 uptake and acetylene reduction activities occurred at exponential and early stationary phase. All data are consistent with BTAi1 as a photosynthetic Rhizobium. The effects of light on nitrogenase activity in the free-living organism will be reported.

C-06

Hydrogen recycling and nitrogen fixation in *Azorhizobium caulinodans* ORS571: Cloning and characterization of *hup* genes. U. Hilgert*, W. de Vries°, M. Ferdinandy-van Vierken°, A. H. Stouthamer°, J. Schell, and F. J. de Bruijn. Max-Planck-Institut, 5000 Koln 30, F.R.G. and °Vrije Universiteit, Amsterdam, The Netherlands.

The reduction of dinitrogen by nitrogen fixing organisms is a highly energy consuming process. The evolution of molecular hydrogen by the nitrogenase represents additional loss of energy. Thus, hydrogen recycling capacity has been assumed to be an advantageous feature of members of the *Rhizobiaceae*. *Azorhizobium caulinodans* ORS571, able to reduce dinitrogen in stem- and root nodules on its host plant *Sesbania rostrata*, as well as in the free living state expresses a hydrogen uptake (HUP) system. To examine the relevance of this system for the energetics of free living as well as symbiotic nitrogen fixation, HUP^- Tn5 insertion mutants of ORS571 were generated and analyzed. In order to isolate the corresponding *hup* gene(s) the mutated region of one of the mutants was cloned and used as a probe to identify the wild type hup sequences. Three clones were isolated, each of which was able to complement the HUP^- phenotype of the mutants. It was shown that these clones share a high degree of homology with hup DNA of *Rhizobium leguminosarum*. The effect of distinct hup:Tn5 mutants on the energetics of free-living nitrogen fixing cultures was determined in the chemostat and the effect on symbiotic nitrogen fixation *in planta*.

C-07

Some characteristics of H_2-uptake and C_2H_2-reduction of Rhizobium arachis in symbiotics state. Xu LiangShu*, Zhang FengZhang, Zeng Ding. Department of Biology, Xiamen University, Xiamen, Fujian, CHINA.

All of the nodules which inoculated with Rhizobium arachis HUP^+ strains (L_{8-3} and F_{I4-2}) and HUP^- strains (C_4 and C09) showed H_2-uptake activity. In full bloom stage, both H_2-uptake and C_2H_2-reduction activities were the highest among the different growing stages. The H_2-uptake activity depended on O_2 and drastically inhibited by carbon monoxide in nodules.

A 48-hrs-dynamic of H_2-uptake and C_2H_2-reduction from the nodules has been obtained. After detachment, nodules were placed in room temperature and kept in proper moisture and the H_2-uptake and C_2H_2-reduction activities were measured respectively at different times. The results showed that C_2H_2-reduction activity decreased gradually during 24-hrs course. But the H_2-uptake activity increased gradually during 24-hrs course and reached maximum at the end. After which, H_2-uptake activity decreased gradually. However, the increase of H_2-uptake activity during the 24-hrs course did not appear when nodules were previously treated with 0.05M succinate or pyruate.

C_2H_2-reduction activity in nodules was significantly promoted by H_2. In the presence of I0% H_2, the C_2H_2-reduction activities of the nodules detached for 3 hrs and 24 hrs increased 20% and 60% respectivity; when treated with 0.05 M succinate, the activity of nodules detached for 24 hrs only increased 30%.

From the evidences obtained, there is a close relationship between the H_2-uptake and nitrogen fixation in peanut plant.

C-08

H-ATPase of the peribacteroid membrane in yellow lupin root nodules. G.I. Livanova*, P.N. Dubrovo, V.V. Krylova, S.F. Izmailov, K.A. Timiriazev. Institute of Plant Physiology, USSR Academy of Sciences Botanicheskaya 35, Moscow, 127276, USSR

The peribacteroid membrane (PBM) of lupin root nodules, like many other biological membranes, has shown to have a transport system translocating protons and capable of producing a proton-motive force. A Mg-ATP-dependent pH shift inside the PBM vesicles isolated from lupin root nodules was demonstrated with acridine orange, a penetrating pH-indicator. The activity of the proton pump was inhibited with orthovanadate and had the pH optimum at 5.3 which was identical to that of the PBM phosphohydrolase activity. Thus, a H-ATPase of a plasma membrane type is suggested to function at the PBM as a proton pump.

C-09 Nickel is essential for active hydrogenase in free-living *Frankia* isolated from *Casuarina*. A.Sellstedt* and G.D. Smith, Dept of Biochemistry, Faculty of Science, Australian National University, GPO, Box 4, Canberra, ACT 2601, Australia.

Five free-living *Frankia* strains isolated from *Casuarina* were investigated for occurrence of hydrogenase activity. Also, nitrogenase activity (acetylene reduction) and hydrogen evolution were evaluated.
Acetylene reduction was detected in the *Frankia* strains. None of them had any hydrogenase activity, when grown on nickel-depleted medium, and they all released hydrogen in air.
After addition of nickel to the medium, the *Frankia* strains were shown to possess an active hydrogenase, which resulted in hydrogen uptake and loss of hydrogen evolution.
The results show that absence of hydrogenase activity in free-living *Frankia* isolated from *Casuarina* is caused by nickel deficiency. *Frankia* living in symbiosis with *Casuarina* are showing hydrogenase activity, and therefore, the results also indicate that hydrogenase activity is to some extent regulated by the host plant.
Furthermore, the results indicate that these *Frankia* strains are different compared to *Frankia* isolated from *Alnus incana* and *Comptonia peregrina*, where hydrogenase activity was detected without addition of nickel.

C-10 **Characterization of the Aerobically Purified Hydrogenase of Azotobacter vinelandii.** SUN Jin-hua and Daniel J. ARP. Laboratory for Nitrogen Fixation Research and Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331.

The hydrogenase of Azotobacter vinelandii has previously been purified under anaerobic conditions. We have now purified the enzyme under aerobic conditions. Hydrogenase was purified to near homogeneity with the same procedure as that used in the anaerobic purification, but the yield was only about 10% of that obtained in the anaerobic purification. This aerobically purified enzyme is active in the $^3H_2$ exchange, H_2 evolution and H_2 uptake (methylene blue as electron acceptor) assays, but the activity can be increased by 2-3 fold by pretreatment with H_2 or dithionite. The aerobically purified hydrogenase showed two significant absorption peaks (450 and 345 nm) in difference spectra of the isolated vs. activated enzyme. An additional peak (315 nm) appeared upon treatment of the activated hydrogenase with O_2. Upon pretreatment of the aerobically isolated hydrogenase with H_2, the increase in activity was correlated with a decrease in absorption peaks at 435 and 345 nm. Activation of the hydrogenase by H_2 was not inhibited by C_2H_2; however, treatment with cyanide did lead to a time-dependent loss of the ability of H_2 to activate hydrogenase. Once activated, aerobically purified A. vinelandii hydrogenase is similar to anaerobically purified hydrogenase with respect to its catalytic properties (e.g. Km for methylene blue, response to pH changes and the effects of O_2).

C-11 Hydrogen-deuterium exchange studies of the two "iron-only" hydrogenases from *Clostridium pasteurianum*. D. Bonam* and L. E. Mortenson. Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602.

The HD exchange technique allows the analysis of hydrogenase enzymes in the absence of net electron flux. D_2 in H_2O is converted into a mixture of D_2, HD, and H_2. The rates and ratios of formation of these products provide information about the active-site of the enzyme. We have studied the bidirectional hydrogenase (H_2ase I) and the uptake hydrogenase (H_2ase II) from *Clostridium pasteurianum*. Although these enzymes are postulated to contain similar hydrogen activating centers (H-centers), they did not show similar HD exchange. H_2ase I catalyzed total exchange at a rate 200 times faster than H_2ase II. Additionally, the H_2 to HD ratio was 0.43 for H_2ase I, but fully 2.2 for H_2ase II assayed under identical conditions. These results suggest that either 1) the H-centers are different, or 2) the ferredoxin-like centers (F-centers) present in these enzymes are indeed involved in HD exchange and fundamentally influence the reaction occurring at the H-center.

C-12 **Localization of the reversible hydrogenase in cyanobacteria.** T.Kentemich* and H.Bothe. Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41

The reversible hydrogenase of the cyanobacterium Anacystis nidulans was purified 250fold by classical methods. Activity staining on gels obtained by native PAGE allowed to identify two bands. Antibodies were raised against the electrophoretically homogeneous protein.The molecular weight of the hydrogenase was determined by SDS-PAGE followed by Western blot analysis with these antibodies. Crude extracts from the unicellular Anacystis and from heterocysts and vegetative cells of Anabaena variabilis showed precipitation bands of 56 and 17 kD. The reversible hydrogenase appeared to be composed of a larger and a smaller subunit as the NiFeS-hydrogenases from other bacteria. The hydrogenase subunits, particularly the smaller one, were subject to proteolysis. By application of the protein A-immuno-gold labeling technique the reversible hydrogenase was shown to associate with the cytoplasmic membrane in Anacystis. It is concluded that Anacystis has two different hydrogenases: the reversible or bidirectional hydrogenase which is located exclusively at the cytoplasmic membrane of the cells, and a thylakoid-bound enzyme which catalyzes only the uptake of H_2. The experiments are published in: Kentemich,T.; Bahnweg, M.; Mayer, F.; Bothe, H., Z.Naturforsch. 44c, 384-391 (1989).

C-13

Effect of monochromatic lights on nitrogen fixation and hydrogen evolution in the isolated heterocysts of Anabaena sp. strain CA. D.Kumar* and H.D.Kumar.Centre of Advanced Study in Botany, Banaras Hindu University,Varanasi 221005, India

Photostimulation of nitrogen fixation (acetylene reduction) and nitrogenase catalysed hydrogen evolution was investigated using monochromatic lights in the isolated heterocysts from N9C (a hydrogen uptake less) mutant strain of Anabaena CA. Nitrogenase activity was strictly light-dependent and fluorescent light showed maximum activity followed by 620 and 650nm monochromatic lights respectively.Isolated heterocysts under fluorescent light showed biphasic curve of hydrogen production. However, when the heterocysts were subjected to monochromatic (620 and 650nm) lights, the curve changed to monophasic without any initial burst. Fluorescence emission spectra were also studied and discussed for energy transfer from phycobilisomes to chl a.

C-14

A research on N_2-fixation and H_2-production of immobilized Anabaena azotica HB686. *Cheng Shuangqi, Chen Zhaoping and Mo Ximu. Dept. of Biology, South China Normal University, Guangzhou 510631, P.R.China

The technique of immobilized cell has made great progress recently. However, there are few reports on applying it to researching N_2-fixation. Now we apply this technique to researching nitrogen fixation of nitrogen fixing micro-organism. Using Na-alginate as carrier, Anabaena azotica HB686 is embedded in Na-alginate to make a particle with 4mm diameter and incubated in free nitrogen medium, estimating the acetylene reducing activity in immobilized and native cell. The acetylene reducing activity in them is 300nmolC_2H_4/mgprotein.hr and 200nmolC_2H_4/mgprotein.hr respectively. The amount of H_2-evolution is 50nmolH_2/mgprotein.hr and 25nmolH_2/mgprotein.hr respectively. The H_2-uptake in immobilized cell is 2.5 times higher than that of native cell, illustrating the energy recycle in immobilized cell to be better than that of native cell. Tolerance to oxygen for immobilized cell is stronger than that of native cell. The acetylene reducing activity of the former decline 25% while the latter decline 45% under 10% of oxygen for 2 hours. The acetylene reducing activity in immobilized cell rise progressively to 300nmolC_2H_4/mgprotein.hr at the 10th day and maintain to the 20th day, but it reaches 200nmolC_2H_4/mgprotein.hr in native cell at the 4th day and decline progressively to 100nmolC_2H_4/mgprotein.hr at the 10th day. These results will provide a base for the industrial ammonia production of N_2fixing micro-organism.

C-15 **Structure of the soybean nodule cortex in relation to oxygen diffusion and regulation of nodule activity.** Richard Parsons[*1] and David Day[2]. [1]Biological Sciences, University of Dundee. Dundee, DD1 4HN, UK. [2]Botany Dept, Australian National University. GPO Box 4 ACT, Australia.

The nodule cortex provides a resistance to the flux of oxygen into the central bacteroid containing tissue and it is often postulated that this resistance is involved in the regulation of nodule activity. This poster presents a detailed analysis of the structure of cells in the cortex, concentrating on the layers of cells where the major resistance to the diffusion of oxygen occurs. In most of the cortex, intercellular gas-spaces are present between cells and calculations presented show that diffusion through these spaces is not the major resistance to oxygen flux.

The major resistance to oxygen movement is apparently formed by a layer of cells in the inner cortex, between which radially orientated intercellular spaces do not occur. This layer of cells (labelled the boundary layer) may surround the entire infected zone of soybean nodules and effectively isolate internal intercellular gas-spaces from cortical gas-spaces and the external air. Micrographs of sections through this boundary layer, orientated in both radial and tangential directions, are presented. The calculated resistance formed by this layer matches the resistance required to maintain the gas concentrations and metabolic rates which are observed in soybean nodules. Furthermore, a role for this boundary layer in regulating oxygen diffusion and thus nodule activity is proposed and a mechanism showing how this regulation may be achieved is presented.

C-16 Measurement of nodule diffusion barrier resistance and respiration rate by *in vivo* spectrophotometry of leghemoglobin. R. F. Denison* USDA, Beckley, WV 25802, USA; and D. B. Layzell, Biology Dept., Queen's Univ., Kingston ON K7L 3N6, Canada.

A recently-developed spectrophotometric method measures fractional oxygenation of leghemoglobin ($fLHGO_2$) in intact, attached nodules under ambient light. This method has previously been used to determine infected-cell O_2 concentration, O_i, which was reported to decrease in response to various types of stress. Changes in $fLHGO_2$ reflect influx of O_2 through a diffusion barrier in the nodule cortex minus O_2 consumption by respiration. Therefore, the rate of decrease of $fLHGO_2$ in a nodule surrounded by 100% N_2 gas can be used to determine respiration rate as a function of O_i. Similarly, the rate of increase in $fLHGO_2$ after an increase in external pO_2 can be used to calculate the resistance of the diffusion barrier, after correcting for respiration. The method will be illustrated using data for soybean (*Glycine max*) and birdsfoot trefoil (*Lotus corniculatus*).

C-17

REGULATION OF O_2 CONCENTRATION IN PEA NODULES.

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The large ATP demand of nitrogenase in legume nodules is met by aerobic respiration, yet nitrogenase is O_2-labile. In soybean nodules infected cell O_2 concentration (Oi) is regulated by a variable barrier to O_2 diffusion in the inner cortex which responds to changes in both external pO_2 and phloem supply . Pea nodules, being indeterminate, are structurally different from soybean nodules and may regulate O_2 in a different manner. Most studies to date with pea have used detached nodules or nodulated roots and have measured nitrogenase activity by the C_2H_2 reduction assay. Unfortunately, these treatments cause nitrogenase inhibition and therefore they are difficult to interpret.

In this study nitrogenase activity and respiration in attached nodulated roots of pea was measured by H_2 and CO_2 production, respectively, in an open flow gas exchange system. Measurements were made on control and stem girdled plants at different external pO_2. The results suggest that nitrogenase in pea nodules may be O_2-limited under ambient conditions, but nodule resistance to O_2 diffusion may increase further in response to phloem deprivation. These results are discussed in relation to direct spectroscopic measurements of Oi, and compared to previous studies with soybean nodules.

C-18

AN OSMOTIC MECHANISM FOR DIFFUSION BARRIER REGULATION IN SOYBEAN NODULES.

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Soybean nodules protect nitrogenase from O_2 inactivation by regulating the resistance of a barrier to O_2 diffusion in the nodule inner cortex. This barrier is thought to consist of a layer of cells with intercellular spaces that are either open or contain a variable depth of water, the amount of water determining the resistance of the barrier. The following evidence supports our hypothesis that sucrose acts as an osmoticant to regulate intercellular water content and thereby diffusion barrier resistance: (a) When nodules were adapted to high pO_2, their diffusive resistance increased and sucrose concentration declined. The pool sizes of 37 other sugars, amino acids and organic acids did not change significantly. (b) After exposure to $Ar:O_2$ nodule resistance increased and nodule osmolarity and sucrose concentration declined. (c) A reduction in phloem supply to the nodule increased resistance and reduced sucrose concentration. (d) A mathematical model indicates that the observed changes in sucrose concentration and osmolarity are sufficient to cause the redistribution of water in the nodule cortex necessary to produce the observed changes in nodule resistance.

Since most of the nodule's starch is localised in the inner cortex, we propose that the nodule controls diffusion barrier resistance by an osmotic mechanism involving regulation of starch/sucrose interconversion.

C-19 THE MECHANISM OF O2-INDUCED INHIBITION OF NODULE METABOLISM IN SOYBEAN.

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When attached, undisturbed soybean nodules are exposed to a step increase in pO2 from 20 to 30%, CO2 evolution and nitrogenase activity (H2 evolution) decline rapidly, but recover with oscillations in activity over the next 30-45 min. The short recovery time indicates that the decline in nodule activity was not due to irreversible nitrogenase inactivation. *In vivo* spectroscopic studies (King et al. 1988, Plant Phys. 87: 296-299) have shown that the infected cell O2 concentration (Oi) increases sharply following the increase in external pO2, but recovers to the initial low level within 8 min.

We propose the following explanation for the observed decline in nitrogenase activity: (a) the rapid increase in Oi allows O2 to compete effectively with nitrogenase for electrons or reduced substrates, (b) the available reductant declines, the ATP increases and nitrogenase activity is consequently inhibited, (c) carbohydrate metabolism is initially stimulated by the increase in Oi, but becomes rapidly inhibited by the accumulated ATP pool, (d) an O2 diffusion barrier acts to regulate Oi, (e) the oscillations in the recovery of nitrogenase activity occur as the pool sizes of metabolites readjust to their initial steady-state levels prior to the O2 treatment. To test the hypothesis , levels of a number of respiratory metabolites were monitored in nodules at various times after exposure to 30% O2. The results will be discussed in relation to the pathways and regulation of nodule metabolism in soybean.

C-20 NITROGEN FIXATION IN LOW RHIZOSPHERE pO_2 LUCERNE.

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In the last years several papers have been published dealing with the effect of oxygen on the nitrogen fixation being the general conclusión that nitrogenase activity could be oxygen-limited, as ARA is enhanced at supra-ambient oxygen levels. However, pO_2s greater than 0,21 atm probably do not occur naturally, while subambiént pO_2s exist under various environmental circunstances.

Plants of lucerne inoculated with **Rhizobium meliloti** were maintained to control (0,21 atm) or low oxygen (0,01 atm) partial pressure in the rooting medium in a long-term experiment in order to ascertain the physiological responses to these anaerobic conditions.

Shoot dry weight was unaffected, but root and nodule DW were reduced by a 50% in this low oxygen level as related to control plants. However, specific ARA showed similar values in both sets of plants, although 1% oxygen-grown plants were more sensitive to acetylene supply. It is suggested that nitrogenase activity in low oxygen conditions could be supported by morphological changes, involving aerenchyma formation in the nodule cortex, facilitating oxygen diffusion and by a more efficient recycling of CO_2.

C-21

Effects of acetylene and external oxygen concentration on RQ of legume nodules. F.R. Minchin* and J.F. Witty. Dept. of Environmental Biology, Welsh Plant Breeding Station, Aberystwyth, Dyfed SY23 3EB, UK.

Carbon dioxide output and oxygen uptake by intact nodulated roots of soybean were determined over a range of external O_2 concentrations (10 to 60%) in the presence and absence of 10% C_2H_2. The responses of root systems were in agreement with the known characteristics of the variable oxygen diffusion resistance of the nodules. These measurements were used to calculate RQ values for nitrogenase-linked respiration at each external O_2 concentration. In the absence of acetylene, RQ values showed no significant response to O_2, with a mean value of 1.08. The addition of C_2H_2 caused a small initial reduction in RQ. It is concluded that measurements of CO_2 production with an IRGA and conversion to values of O_2 uptake using an RQ of 1 is the best routine experimental procedure for obtaining comparative estimates of diffusion resistance. It is also concluded that changes in diffusion resistance are largely due to physical, rather than biochemical, processes.

C-22

C_2H_2 reduction by broadbean nodules and bacteroids under water stress : role of oxygen. J.C. Trinchant*. V. Guérin and J. Rigaud. Lab. Biologie Végétale et Microbiologie. URA C.N.R.S. 1114. Faculté des Sciences, Nice, France.

Broadbeans (*Vicia faba* L.) exhibited decreasing leaf and nodule water potentials linearly and highly correlated, throughout the water deprivation period. A decrease of 0.2 MPa of the nodule water potential (ψ_{nod}) induced a 25 % inhibition of C_2H_2 reduction activity of nodules. This activity continued to be depressed when water stress increased but the effect was less pronounced. In contrast, a total recovery of C_2H_2 reduction activity of broadbeans submitted to water stress for 4 days (ψ_{nod} = -1.6 MPa) occurred 6 days after rewatering. Partial recovery of N_2 fixation of midly water stressed plants was possible by increasing the external pO_2 up to 60 KPa. The dense packing of the cortex cells of stressed nodules may be responsible for the limitation of O_2 diffusion. When bacteroids were isolated from broadbean nodules, optimal C_2H_2 reduction activity occurred for pO_2 higher than those generally observed with other legumes. Glucose was 2.8 times more efficient than succinate to support bacteroid C_2H_2 reduction. This activity declined with increasing nodule water deprivation, optimal capacity always requiring lower O_2 tensions in direct relation to a limitation of the bacteroid respiration. Water stress was also responsible for a drop in the cytosolic protein of nodules and more specifically leghemoglobin, affecting the O_2 availability for the bacteroids.

C-23

Possible mechanism of the acetylene-induced decline in the actinorhizal nodules of Myrica gale. J.D. Tjepkema* and C.R. Schwintzer*, Dept. of Botany & Plant Pathology, University of Maine, Orono, ME 04469, USA.

During the period of from 1 to 3-5 minutes after acetylene addition, a peak rate of ethylene formation is observed. The rate then declines to values as low as 10% of the peak rate and then recovers to between 50 and 80% of the initial peak rate over a period of about 60 minutes. We hypothesize that nitrogenase turns over more rapidly when acetylene is being reduced than when N_2 is being reduced. The introduction of acetylene may thus lead to the depletion of the pool of reductant used by nitrogenase, with a subsequent decline in the rate of ethylene formation. A much smaller decline was observed in assays at 5% O_2, where nitrogenase activity is strongly oxygen limited. Under these conditions the rate of acetylene reduction is likely to be more limited by the rate of ATP formation than by the rate of reductant formation. After inactivation of most of nitrogenase by exposure to high pO_2, no decline is observed following acetylene addition. Under this condition there should be a large surplus of reductant, so the lack of a decline supports our hypothesis. We have also examined the effect of pO_2 on acetylene reduction during the period of the minimum rate. Decreasing the pO_2 during this period increases acetylene reduction activity. This increase may be due to relief of competition for reductant between nitrogenase and ATP formation during the decline phase.

C-24

Ferric leghemoglobin reduction in legume nodules M. Becana* and R. V. Klucas, Department of Biochemistry, the University of Nebraska-Lincoln, NE, 68583-0718

The maintenance of leghemoglobin (Lb) in the ferrous form (Lb^{+2}) is a prerequiste for nitrogen fixation since ferric Lb (Lb^{+3}) is unable to bind O_2. Evidence is presented for the operation in legume nodules of at least three systems for the restoration of Lb^{+2}. (i) Reduction of Lb^{+3} by a leghemoglobin reductase in soybean nodules. The enzyme is a FAD-containing protein, has an Mr of 99-109 kD and two equally-sized subunits, and exhibits Km values of 9 μM for soybean Lba^{+3} and 51 μM for NADH. NADPH is only 30% (initial velocities) as effective as NADH. Pure leghemoglobin reductase converts 215 nmol Lb^{+3} to Lb^{+2}-CO (or Lb^{+2}-O_2) per min per mg protein, and does not require an exogenous electron carrier. The enzyme shows similar affinity for soybean, bean and cowpea Lbs^{+3}, but different maximum velocities. The reductase is inactive when Lb^{+3} is bound to nicotinate or NO_2^-. (ii) Direct reduction of Lb^{+3} by NAD(P)H, ascorbate and cysteine. (iii) Reduction of Lb^{+3} by NAD(P)H-reduced flavins; a reaction that does not involve O_2^- or H_2O_2 and proceeds more efficiently according to the order riboflavin > FMN > FAD.

C-25 Oxygen requirement for the enzyme-catalyzed oxidation of NADH and reduction of ferric leghemoglobin.

R. V. Klucas* and L. Ji, Department of Biochemistry, the University of Nebraska-Lincoln, NE, 68583-0718

Ferric leghemoglobin reductase (FLR), an enzyme hypothesized to maintain leghemoglobin in soybean nodules in the functional ferrous state, was purified to homogeneity. The pure protein has FLR and DCPIP reductase activities and requires a reductant (NADH or NADPH) for activity. O_2 is required for NADH oxidation as well as for ferric leghemoglobin (Lb^{+3}) and DCPIP reductions. Enzymic activities for NADH oxidation, Lb^{+3} reduction and DCPIP reduction are completely inhibited by removing O_2 and restored by addition of O_2. Peroxidase or catalase at low concentration (about 2μg) stimulate oxidation of NADH and reduction of Lb^{+3}, suggesting that peroxide intermediates are involved in the reaction. Stoichiometry of the enzyme-catalyzed oxidation-reduction of NADH-Lb^{+3} is not constant but varies with time and different concentrations of NADH. A partial sequence of the 50 N-terminal amino acids of the purified enzyme was obtained. The computer-based comparative studies with known protein sequences showed that the sequence of this enzyme is highly homologous to those of the flavin-nucleotide disulfide oxidoreductases, especially lipoamide dehydrogenase. A mechanism for this enzyme-catalyzed oxidation of NADH and reduction of Lb^{+3} is proposed.

C-26 Poly-β-hydroxybutyrate and N_2 fixation by soybean bacteroids.

F.J. Bergersen*, C.S.I.R.O. Plant Industry, Canberra, A.C.T. 2601, Australia.

Accumulation and utilization of ^{14}C assimilated by soybean bacteroids was studied in a stirred reaction chamber supplied with a steady flow of medium containing [U^{14}C]-malate, oxyleghaemoglobin and dissolved air or the same medium with no malate. Spectrophotometry of the effluent stream enabled calculation of O_2 concentration and consumption. N_2 was fixed to NH_3. Samples of bacteroids from the chamber and CO_2 dissolved in the effluent stream were analyzed for ^{14}C.

Principal results were:- (1) At very low concentrations of free O_2 in the chamber, ^{14}C from malate accumulated in the bacteroids, ^{14}C-poly-β-hydroxybutyrate (PHB) was synthesized, ^{14}C-CO_2 efflux was high and N_2 fixation low.

(2) After washout of extracellular malate, N_2 fixation increased greatly; PHB continued to be synthesized from stored intracellular ^{14}C.

(3) After exhaustion of intracellular non-PHB-^{14}C, a new, PHB-utilizing steady state was established and N_2 fixation remained high. Low ^{14}C in respired CO_2 reflected the large amount of stored PHB already present when the experiment began.

C-27

Release of bacteroid proteins during the preparation of symbiosome membrane and symbiosome space fractions from soybean nodules. J.G. Streeter*, Dept. of Agronomy, Ohio State Univ./OARDC, Wooster, OH 44691.

Marker enzymes for the symbiosome membrane and symbiosome space are required as guides during the purification of these components from legume nodules. In preliminary work on the development of a system for the preparation of highly purified membrane and space fractions, we evaluated various techniques for handling and rupture of symbiosomes from Glycine max nodules in an attempt to minimize contamination of membrane and space fractions by bacteroid proteins. Symbiosomes were purified from soybean nodules on discontinuous Percoll gradients. Phosphodiesterase was used as a periplasmic marker and β-hydroxybutyrate dehydrogenase was used as a cytoplasmic marker for bacteroid proteins. Osmotic shock, which is commonly used as a technique for rupturing symbiosomes, released substantial phosphodiesterase activity (as much as 55% of total activity) and a smaller but significant amount of β-hydroxybutyrate dehydrogenase from bacteroids. Extrusion of symbiosomes through a fine needle (I.D. of 0.11 mm) is the recommended rupture treatment, and this treatment released much less phosphodiesterase and almost no β-hydroxybutyrate dehydrogenase from bacteroids. α-Mannosidase was detected in the symbiosome space fraction but only under conditions where β-hydroxybutyrate dehydrogenase was also found. Thus, α-mannosidase does not appear to be a marker for the symbiosome space as reported by others.

C-28

Rapid release of $^{14}CO_2$ during incubations of Bradyrhizobium japonicum bacteroids with ^{14}C-labeled metabolites. S.O. Salminen* and J.G. Streeter. Dept. of Agronomy, O.A.R.D.C./Ohio State University, Wooster, OH 44691, USA.

Previous studies have indicated that release of $^{14}CO_2$ varies widely when various ^{14}C-labeled metabolites are supplied to bacteroids during incubations of 20 to 60 minutes (J. Bacteriol. 169(1987)495). We wondered if $^{14}CO_2$ release was significant during the shorter incubations (<5 min) generally used to estimate Km and Vmax values for uptake of various substrates. The uptake of metabolites by the bacteroids was measured by the silicone oil centrifugation technique (J. Bacteriol. 159(1984)47), and $^{14}CO_2$ released was trapped using a paper wick impregnated with 100 µl of 10% KOH. We found that (1) most of the $^{14}CO_2$ released was retained by the incubation medium and was quantitatively released only after acidification of the reaction mixture and shaking for 10 min; (2) release of $^{14}CO_2$ began immediately after mixing bacteroids and ^{14}C-labeled substrate, and amounted to as much as 40% of total substrate taken up after only 1 min; (3) the rate of conversion of substrate to $^{14}CO_2$, as a proportion of total uptake, varied among substrates; and (4) failure to account for $^{14}CO_2$ released could result in underestimation of Vmax by as much as 50%.

C-29 Low [O_2] is the probable cause of α,α-trehalose accumulation in legume nodules. I. Hoelzle* and J.G. Streeter, Department of Agronomy, Ohio State University/OARDC, Wooster, Ohio 44691, USA.

Trehalose is synthesized in bacteroids and accumulates rapidly at the onset of N_2 fixation in legume nodules. We wondered if trehalose accumulation might be related to the microaerophilic conditions found in infected cells and tested this hypothesis by comparing the accumulation of trehalose in cultured rhizobia grown with 1% or 21% O_2 in the gas phase. Rhizobia cultured with the reduced O_2 concentration accumulated significantly more trehalose. A similar response was obtained when different C sources were used (glycerol, glucose, succinate or complex media), and with 4 different rhizobia (Rhizobium leguminosarum bv. phaseoli USDA 2667, R. l. bv. viceae USDA 2391, R. fredii USDA 191, R. meliloti USDA 1021). Increases in trehalose concentration (μg trehalose/mg protein) under low O_2 tension ranged from 8-fold to more than 40-fold, depending on the strain of rhizobia and the culture medium composition. An experiment in which R. l. bv. phaseoli USDA 2667 cells were grown under 1% O_2 and then switched to 21% O_2 demonstrated the ability of rhizobia to accumulate trehalose under O_2 stress and then rapidly metabolize it when O_2 levels increased. The marked accumulation of trehalose under low [O_2] provides additional evidence that this disaccharide, which is found in all N_2 fixing symbioses, may have some as yet unknown function in the fixation process.

C-30 Glutamine synthetase may limit nitrogen fixation in Phaseolus vulgaris. R. S. Pacovsky* and M. Hungria, USDA-ARS-WRRC, Albany, CA 94710 and University of California, Davis, CA 95616

Previously bacterial nitrogenase or host photosynthesis was assumed to be responsible for the low net N_2 fixation by Phaseolus vulgaris. In 3 separate experiments, N_2 fixation and specific nodule activity (SNA) were measured using either N balance, C_2H_2 reduction with H_2 evolution or 15-N methodologies. Protein extracts were prepared from nodules of 3 cultivars of field beans, some responsive and some less-responsive to nodulation, that were inoculated with 4 different Rhizobium leguminosarum bv. phaseoli strains. Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities were low relative to each SNA. Transferase/synthetase ratio for GS was also low indicating that the synthetase activity was saturated and operating at maximal level. GS and GOGAT activities preceeded nitrogenase function and increased as the symbioses developed. SNA and the level of GS were significantly correlated ($r = 0.90^*$) for each cultivar at each harvest. GOGAT activities on a mg protein basis were significantly lower in the less-responsive cultivars, but this may be due to the decreased N input in these nodules. GS activity depended not only on the Phaseolus cultivar but also on the strain of Rhizobium, indicating that host gene expression can be modified due to the microsymbiont. This suggests that assimilation of N by GS is the rate-limiting step in N_2 fixation by these bean cultivars with different symbiotic efficiencies.

C-31 Function of nitrogenase in Rhizobium meliloti bacteroids. R.W. Miller, Plant Research Centre, Agriculture Canada, Ottawa, Canada, K1A 0C6.

R. meliloti bacteroids isolated from alfalfa nodules supported nitrogenase activity only in the presence of added succinate or L-malate. Respiration-supported nitrogenase was 1/4 to 1/3 of total nitrogenase activity present in the bacteroids. Total nitrogenase activity was determined by acetylene reduction following treatment of bacteroids with chlorpromazine. Treated cells were assayed with external reductant and an MgATP generating system. Chlorpromazine eliminated coupling between respiration and nitrogenase and rendered the cell membrane permeable to dithionite and nucleotides but did not inhibit the soluble nitrogenase system. ATP/ADP ratios (measured with intact bacteroids) limited nitrogenase activity in vivo above 20°C to a fraction of the total activity. Dilution effects on proton reduction by the cell-free bacteroid nitrogenase system indicated that the excess of active Fe protein over MoFe protein was not more that 2 fold in unpurified extracts. The R. meliloti MoFe protein (Rm1) was purified to a specific activity of 1400 nmoles H_2 per min. per mg. Rm1 activity was fully complemented by Ac2 and Kp2 as well as Rm2.

C-32 Purification of a Cytochrome c Oxidase from Bradyrhizobium japonicum Bacteroids by Lectin Affinity Chromatography. R.G. Keefe and R.J. Maier. Dept. of Biology, the Johns Hopkins University, Baltimore, MD 21218, USA

Our previous work directed towards purifying a cytochrome c (cyt c) oxidase from Bradyrhizobium japonicum bacteroids showed that this enzyme might be glycosylated. For the most part, purification efforts have been carried out with bacteroids from soybeans inoculated with strain LO501, which is derived from (the parent) strain USDA DES 122. LO501 is a Tn5-induced cyt aa_3 structural mutant as a free-living bacterium, but symbiotically, it also has elevated cyt c oxidase activity compared to wildtype. Using data from a monosaccharide analysis of a partially purified cyt c oxidase sample, experiments were carried out to see if oxidase activity could bind lectin. Three different lectins, each covalently attached to either cross-linked 4% beaded agarose or sepharose, were tested. Of the three, an affinity column made of concanavalin A (con A) covalently attached to sepharose could bind bacteroid cyt c oxidase activity. This activity could be eluted from the con A column with 0.3 M methyl-mannose. When samples from peak activity fractions from a con A column were run on SDS polyacrylamide gels and silver-stained, three protein bands were observed. Their molecular weights were roughly calculated (using SDS PAGE, 15% resolving gel) to be 25.6, 24.4 and 13.5 kdal. The two larger subunits are visible on denatured gels using the periodic acid-Schiff base (PAS) stain which is specific for carbohydrate. The 13.5 kdal band was not stained with PAS. Total protein solubilized from the membranes of free-living LO501 bacteria, run on denatured gels and stained for glycoproteins showed no visible bands, whereas the same protein sample from LO501 bacteroid membranes showed only the ~25 kdal doublet using PAS.

SESSION D

Nitrogen fixation and the environment
D-01 - D-54

D-01

Effect of BNF on the elemental concentration of M.s. grown under various salinity levels. Catherine E.Douka*, A. C. Xenoulis, T. Paradellis. Inst. of Biology NRCRS"Demokritos" Ag. Paraskevi; 15310 Athens Greece.

A salinity tolerant Rhizobium meliloti isolated from a Greek soil was used to inoculate Medicago sativa plants grown in Nitrogen-Free Agar medium in presence of 0, 0.2, 0.4, 0.8, and 1.2% NaCl. In the upper part of the plants, grown for 3 months with and without inoculation under the various salinities, the concentration of various elements was determined by using the X-ray fluorescence technique. The concentration of several elements, such as Mo, Mn, Ca, Sr, Ni, Zn and Cu, in the protein produced via Biological Nitrogen Fixation was found significantly redused compared to that in the uninoculated plants. The degree of reduction varies with the levels of salinity. The growth of the plants and the degree of Biological Nitrogen Fixation (dry matter yield and number of nodules per plant) as well as the difference in the elemental concentration between inoculated and uninoculated plants, decreases with increasing level of salinity in the growth medium. At 1.2% NaCl insignificant growth of the plants occured.
The relationship between Biological Nitrogen Fixation and plant elemental concentration as influenced by the different salinity levels will be discussed.

D-02

Effect of 1.5% NaCl on Medicago sativa - Rhizobium meliloti symbiosis.

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Two symbiotic systems differing in their NaCl-tolerance -alfalfa cv. Khivinskaya (Kh) and NaCl-tolerant (up to 2% NaCl) line 124 produced by cell selection from Kh were compared. Plants were inoculated with an effective Rh.meliloti 225 strain tolerant to NaCl up to concentration 3%. The plants were grown on N-free Lie nutrient solution without NaCl for 2 weeks, and than for 6 weeks with or without 1.5% NaCl. Dry weight of shoots of Kh. plants was only 31% of control while that of line 124 was 69% of its control. 1.5% NaCl affected the root nodule initiation in both salt-tolerant and salt-sensitive alfalfa plants. Dry weight of nodules was 25% and 21% of control ones respectively. However nodule activity (C_2H_2) in line 124 was similar to activity in line 124 in the absence of NaCl while in Kh. it decreased to 26% under NaCl influence. Decreasing of root nodule activity in Kh. plants was the result of cell structure degradation in nodule central tissue. Conjugation of bacteroids as well as their budding occured in nodules Kh. plants. We suggest that NaCl may produce a direct effect on nodule initiation. Salt tolerance of nodules depends on salt-tolerance of whole plant.

D-03

Response of four alfalfa (Medicago Sativa L.) cultivars to salinity under different N_2 dependent regimes. Y. Kapulnik* and Bruria Heuer, Institute of Field Crops, The Volcani Center, ARO, POE 6, Bet Dagan 50250, Israel.

Forage production of four alfalfa cultivars under saline conditions was investigated. The cultivars tested were Ghilboa, Mohapa 69, Hairy Peruvian and Alic (Turko), originally selected as best in their native countries. Cultivar Turko was outstanding in its response, showing only a slight inhibition of forage production, while all the others showed a significant reduction in growth, up to 50 percent. The different response could not be attributed to a different pattern of ion accumulation, as the increase in Cl^- and Na^+ content in the shoots was identical. Other possibilities were studied and will be discussed. The effect of different nitrogen levels on growth and nodulation under salinity was also investigated. In plants exposed to saline conditions, nitrogen increased growth and development over N_2 dependent plants, but the relative reduction in plants growth was similar. Both nitrogen and salt reduced nodulation and N_2 fixation on a per plant basis, but only nitrogen reduced nodule specific activity.

D-04

Nodulation and nitrogen fixing capacity in relation to variable salinity levels and their dependability on regulators in Cicer arietinum L. (chickpea). I.S. Dua and N. Garg. Department of Botany, Panjab University, Chandigarh - 160014. INDIA.

Experiments conducted to study the effects of salinity on nodulation and nitrogen fixation in Cicer arietinum L., a highly sensitive leguminous crop, revelaed significant variation with regard to its survival in relation to saline environment as measured by electrical conductivity (EC), available in the growing soil culture. It was observed that whereas the EC value of the order of 6 and 8 dSm^{-1} were exclusively lethal, the stress as induced by an electrical conductivity measuring 4 $dS\ m^{-1}$ was comparatively less deleterious. The effects of these stresses were elicitable on nodulation, nitrogen fixation, leghemoglobin content, nitrogen content, peroxidase activity vis-a-vis specific key metabolic changes which directly pointed that salinity-induced detrimental effects were possibly metabolically regulated and besides this, operated through the intervention of some key regulatory substances. A probe into the relative levels of cytokinins namely zeatin, 2 iP, zeatin riboside and zeatin ribotide revealed the presence of lower quantum of these compounds as compared to salt resistant akin crop like Glycine max. Keeping in line with this contention, the exogenous application of these compounds partially upgraded the salinity resistance in the experimental sensitive crop (chickpea).

D-05

Lipopolysaccharide and protein patterns of rhizobia isolated from root-nodules of legumes grown in arid and salt-affected lands in Egypt and the Sudan.

H. H. Zahran* and K. Lindström, Dept. of Microbiology, University of Helsinki, SF-00710 Helsinki, Finland.

Electrophoretic patterns of lipopolysaccharide (LPS) and whole cell protein of 60 strains of rhizobia were analyzed and compared using SDS-PAGE. The rhizobia came from woody, grain and forage legumes grown in arid and salt-affected lands in Egypt and the Sudan. Most of the strains studied were salt-tolerant, being able to grow in medium supplemented with 3% NaCl. The LPS and protein profiles of these strains were altered after growth in 3% NaCl. Alteration of LPS was indicated by changes in colour and density of bands, by disappearance of certain major bands and by formation of aggregates on SDS-PAGE. Alteration of protein profiles was indicated by disappearance of certain bands and appearance of new bands. It is concluded that alteration of protein and LPS synthesis patterns might be a mechanism for adaptation to salt stress. Alteration in LPS structure might inhibit the infectivity and nodulating ability of these rhizobia.

D-06

Efficiency and persistence of alfalfa rhizobia in soil as affected by salinity and desiccation. A.H. Al-Rashidi* and N.Y. Aziz. Soil Dept., University of Basrah, Iraq

The interactive effects of 3 soil salinity levels (5.0, 9.0, &12.0 dS/m), 2 moisture contents (F.C and 1/2F.C.) and 5 rhizobial inoculations on nitrogen fixation by alfalfa (Medicago sativa L.) were assessed in the greenhouse. Analysis of variance indicated that salinity and rhizobial inoculation were primarily affecting acetylene reduction. Moisture content also exerted statistically significant influences. Significant decreases in top growth dry weight and nodule number per plant were also obtained with salinity treatments. Differences in acetylene reduction by rhizobia were found to vary significantly between strains and isolates. Tolerance of rhizobia to osmotic dehydration was studied in YMB adjusted by salt mixture to various salinity (1, 4, 8, and 12dS/m) and in YMB adjusted to various water activities (a_w). The maximum growth observed at salinity levels of 8 and 12 dS/m, was obtained with isolates 2-1 and 10-2 and strain 3D0a 29a. Similar patterns of survival among the strains and isolates in the lowest a_w (0.980) were found with growth on YMB at various salinity. The data presented herein suggest that there is a relationship between better survivability of strains or isolates at salt stress and low water activities medium and the ability of rhizobia to fix nitrogen in soil affected by salinity and low moisture content.

D-07

Colonizing ability of actinorhizal Casuarina glauca under salt-affected and waterlogged conditions in Pakistan. A.H. Chaudhary* & G.R. Sandhu, Quaid-i-Azam University & Pakistan Agricultural Research Council, Islamabad, PAKISTAN.

The twin menace of waterlogging and salinity has plagued agriculture in vast areas of Pakistan. The soils being mostly saline-sodic & fine textured with drainage problems, revegetation with suitable plants has been shwon to be more feasible than the engineering and chemical approaches. Casuarina species have given good performance in coastal saline belts in many countries. Under specific soil & climatic conditions of Pakistan, both laboratory & field experiments have given encouraging results in inland salt-affected and waterlogged areas. The nitrogen fixing capacity of Casuarina glauca is an added advantage. The data presented on growth, nodulation and nitrogen fixing ability both under lab and field conditions confirms the usefulness of this plant as a primary colonizer & preferred biomass producer under the existing harsh conditions. A number of Frankia isolates from Casuarina nodules have also been obtained and characterized.

D-08

CYANOBACTERIAL NITROGEN FIXATION IN SALINE ENVIRONMENTS

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The photoautotrophic N_2 fixers cyanobacteria, display a considerable tolerance to a varietyof environmental stresses, including salinity. Salinity stress however, reduces their diazotrophic efficiency and the reasons for this have been investigated. Exposure to salt (Nacl) stress inhibited N_2 fixation in a salt sensitive strain, Anabaena L. 31 white a salt tolerant strain A. torulosa responded initially with higher rates of fixation followed by inhibition at higher salinities. The effect of osmotic and ionic components of salt stress were distinguished experimentally and showed that both the synthesis and activity of nitrogenase was completely insensitive to purely osmotic stress (sucrose). However, ionic stress in the form of Na^+ (but not K^+) severely inhibited nitrogenase expression. Environmental factors (such as alkaline external pH, high external K^+, etc) which curtailed Na^+ influx substantially enhanced N_2 fixation of both strains irrespective of their inherent sensitivities to salt stress. The data suggest that diversion of energy from N_2fixation to osmoregulatory process is mainly responsible for sensitivity of N_2 fixation to salt stress. Factors which minimise or eliminate the energy requirement of Na^+ extrusion enhance N_2 fixation under salinity. Provision of exogenous combined N which inhibits Na^+ uptake confers maximum osmoprotection by eliminating energy requirement for both osmoregulation and N_2 fixation.

D-09

Diazotrophs Isolated Repeatedly from Roots of Kallar Grass Form a New Genus, AZOARCUS. B. Reinhold-Hurek, T. Hurek, M. Gillis, B. Hoste, K. Kersters, and J. De Ley. Lab. voor Microbiologie and Microbiele Genetika, Rijksuniversiteit Gent, B-9000 Gent, Belgium

A survey of the most abundant N_2-fixing bacteria in roots of Kallar grass (Leptochloa fusca (L.)Kunth) grown on a salt-affected field in Punjab, Pakistan, was carried out in 1984 and 1988. Eleven strains, most of them originating from the endorhizosphere, belong in a new genus, Azoarcus. Cells are gram negative curved to straight rods, motile by one polar flagellum, and contain granules of poly-β-hydroxybutyrate. Colonies are yellowish. They were enriched on malic acid in semisolid nitrogen-free medium, in which they grow microaerobically as a subsurface pellicle. They have a respiratory type of metabolism and are restricted in their use of carbon sources to organic acids and few amino acids and are not growing on carbohydrates. Deoxyribonucleic (DNA) - ribosomal ribonucleic acid (rRNA) hybridizations showed no close relationship to any other known genus, the isolates constituting a seperate rRNA branch in rRNA superfamily III: on this rRNA branch we found two groups with $T_{m(e)}$ values around 80°C and 72°C, respectively. Polyacrylamide gel electrophoresis of cellular proteins, analysis of fatty acid patterns and carbohydrate utilization, and DNA - DNA homology studies revealed five subgroups differing at the species level. Clinical isolates and a strain originating from raffinery-oil sludge could also be assigned to this genus.

D-10

Soil acidity-factor effects on *Bradyrhizobium japonicum* and soybean symbiosis. A.A.T. Vargas[1]*; N.D. Denardin[1]; L.E. Roth[2]; G.L. Sexton[2]. [1]Centro de Energia Nuclear na Agricultura CENA-USP, CP 96, Piracicaba 13400, Brazil; [2]University of Tennessee, M313 Walters Building, Knoxville, TN 37996-0810.

Experiments were conducted in the greenhouse to determine soil acidity effects on the soybean (*Glycine max*) and *B. japonicum* symbiosis. Two cultivars, IAC-9 and IAC-13, were inoculated with 10^9 and 10^{10} cells/jar of CM-1529, an acid-tolerant strain. A modified Leonard jar (Vincent, 1970) system was used in which the only function of the lower container was to retain nutrient solutions voided from the upper chamber. Sterile plant nutrient solutions were added daily in the top part of the jar. Levels of acidity were pH 4.5 and 6.5, aluminum at 50 and 100 μM, and phosphorus at 50 to 100 μM. Both cultivars showed up to 10^9 and 10^{10} bacteroids/g nodule 26 DAP based on direct and pour-plate counts, respectively. Acid pH associated with aluminum toxicity and low phosphorus concentration reduced nitrogen fixation (nodulation and acetylene reduction) of both cultivars but did not affect top-plant dry weight of IAC-9. Concentrations of nutrients in top (N, P, Al) and roots were also affected by acid treatments, with different responses for each cultivar. The jar system used was suitable to detect differences between treatments for most of the parameters evaluated. Light and electron microscopy showed perinuclear digestion vacuoles in infected cells and a lack of starch grains in uninfected cells in nodules of plants grown in acid and aluminum conditions.

D-11

Construction of acid tolerant, nitrogen-fixing strains of *Rhizobium leguminosarum* bv *trifolii*. Chen, H., Gartner, E., Djordjevic, M. A., and Rolfe, B. G.* Plant Microbe Interaction Group, Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia.

Acidification of soils beneath clover-based pastures is a serious problem in southern Australia. Although clover plants themselves are moderately tolerant to acid conditions, *R.l.* by *trifolii* and clover nodulation are sensitive to low pH and associated acid soil factors (1). Most acid-tolerant *R.l.* bv *trifolii* nodulate clover plants at low pH, but are ineffective. Therefore, we sought to construct acid-tolerant *R.l.* bv *trifolii* strains with enhanced nitrogen-fixing capacity.

The acid-tolerant *R.l.* bv *trifolii* strain ANU1173 ineffectively nodulates clovers at pH 4.3. It contains four plasmids, the smallest is the Sym-plasmid which can be mobilized into other strains conferring the Nod+Fix+/- phenotype. The Sym-plasmid of strain ANU1173 can be exchanged with the clover nodulation clovers. When strain ANU1173 can be exchanged with the clover nodulation plasmid pBRIAN which, results in strains that form very effective nodules on clovers. When Strain ANU1173 was cured of its second largest plasmid, it became acid-sensitive (strain p22). A series of Tn5-induced acid sensitive mutants of strain ANU1173 have been cloned. These mutants grew normally at pH 6.5 but very poorly at pH 4.7. Several acid-tolerant loci were identified. Some were a non-Sym-plasmid located and others were chromosomally located. 1. Richardson, A. E. *et al* 1989. *Aust. J. Physiol.* **16**:117-29. 2. Djordjevic, M. A. *et al* 1983. *J. Bacteriol.* **156**:1035-45.

D-12

Nitrogen-rich nodule symbiotic phenotype of Rhizobium leguminosarum bv viciae and its potential application for competition studies. V. Škrdleta^{+}. M. Němcová. L. Lisá. D. Kovářová and K. Novák. Dept. of Ecology of Microorganisms, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, CS 14220, Czechoslovakia.

The nodules which developed on roots of Pisum sativum L. after inoculation with R. leguminosarum strains showing a Hup^{+} symbiotic phenotype and/or a high relative efficiency of electron transfer to dinitrogen (RE) had a high nitrogen content of their tissue. In comparison with the nodules initiated by a strain possessing the Hup^{-} phenotype or by an indigenous soil Rhizobium population, the nitrogen-rich nodules contained up to four times more nitrogen (9,2% of dry mass). The nitrogen-rich nodule symbiotic phenotype (Nrn^{+}) was well expressed irrespective of pea cultivars and host plant age. If the strain showing Nrn^{+} phenotype was applied in double-strain inocula, the significant correlation was found between increasing rates of the strain and increasing percentage N content of nodule tissue in the nodulated plants.

D-13 Nodulation competitiveness of hydrophobic mutants of Rhizobium leguminosarum bv. phaseoli.

Jocelyn L. Milner*, Ricardo Araujo, and Jo Handelsman. Department of Plant Pathology, University of Wisconsin, Madison, WI. 53706 USA

Mutants of Rhizobium leguminosarum bv. phaseoli strains TAL182v, CIAT899, and KIM5s were isolated to investigate the relationship between cell surface hydrophobicity and nodulation competitiveness. Cultures were mutagenized with transposon Tn5 and were subjected to a hexadecane separation to enrich for hydrophobic cells. After 3 rounds of culture growth, each followed by separation of the bacteria in the aqueous and solvent phases, the solvent phase was streaked for single colonies and individual mutants were tested for hydrophobicity. Several independent mutants were obtained from each parent strain. Hexadecane separations of cultures of each mutant resulted in greater clearing of the aqueous layer and formation of a thicker emulsion layer than of the cultures of the corresponding parent strain. All mutants were tested against their parent strains for nodulation competitiveness on bean plants (Phaseolus vulgarus L.). Mixtures of each mutant and its parent were applied to seeds at a ratio of approx. 1:1. In the mixed inoculum experiments, each of the hydrophobic mutants of TAL182v occupied significantly more nodules than did strain TAL182v, whereas the mutants of KIM5s and CIAT899 occupied the same number or fewer nodules than did their parent strain. One hydrophobic mutant, strain TL1026, was applied to seeds with its parent, TAL182v, at several ratios; a 50-fold excess of strain TAL182v was required for it to occupy half of the nodules. In addition, mutant TL1026 outcompeted strain KIM5s, a strain that is more competitive than strain TAL182v. The data indicate that cell surface hydrophobicity may be a factor involved in nodulation competitiveness of some strains. The genetic and biochemical basis for this phenotype, which may differ among the parent strains, is being studied.

D-14 Competitiveness of Rhizobium strains compared to their growth rates under carbohydrate limitation

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The competitiveness of 6 R. leguminosarum bv. phaseoli strains was compared to their growth rates under carbohydrate limitation in a "Dialysis culturing system". In this new developed dialysis culturing system 8 strains can be cultivated under exactly the same conditions down to 10^{-6} M carbohydrate concentration under constant medium exchange. Rhizobium strains with higher growth rates showed higher competitiveness compared to strains with less effective uptake systems. The determination of growth rates under carbohydrate limitation can be used as one screening parameter for competitiveness with a large number of laboratory strains in a short time.

D-15

Survival and competition of Rhizobium leg. bv. phaseoli strains.

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The survival and competition of six Rhizobium phaseoli strains were tested under different climatic conditions and soil types. The most competitive strains KIM 5 and Ciat 895 showed highest nodule occupancy under temperate climatic conditions and soil types of W. - Germany and under the tropical climatic conditions in non acid soils at Cali, Colombia. But the competitivity was affected by the soil pH and the strains mentioned above were outcompeted by acid tolerant strains (Ciat 151 and Ciat 899). Nodule occupancy, nodule number, plant dry weight, acetylene reduction and nitrogen content were investigated at three different stages of plant growth in different soil types.

D-16

Symbiotic performance and competition ability of some spontaneous mutants of Rhizobium meliloti.

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In order to select useful strains as inoculants under different soil conditions and host genotypes, 39 spontaneous mutants of Rhizobium meliloti strains 1107, 1025, 102F97, 102F51A, characterized by resistance to streptomycin or spectinomycin were evaluated for their ability to nodulate seedlings of four cultivars of Medicago sativa under aseptic conditions on agar tubes. The acetylene reduction activity of nodules on six weeks old plants was also evaluated. Some representative strains, selected for the precocity of nodulation and an high number of nodules formed on agar tubes, compared with their wild types, were analyzed for the ability to compete for nodulation in soils were indigenous populations are present, at different rates of inoculum.
The symbiotic performance of some strains analyzed under aseptic conditions on different cultivars were correlated with the competition ability for nodulation in the soil.

D-17

Competitiveness of two effective strains of Rhizobium cowpea I-125 and I-700 in cowpea plants inoculated at two different root levels.

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Seeds of cowpea (Vigna unguiculata (L.) Walp. var. tuy) were planted in sterilized Leonard jars containing vermiculite and 0.8L. of Fahraeus solution devoid of nitrogen. Two strains of R. cowpea, incorporated in gamma irradiated pit were allocated either at 5 cm (up) and/or 15 cm (low) from the upper level of the vermiculite, according to the following treatments: T1) I-125 up; T2) I-125 low; T3) I-125 up and low; T4) I-700 up; T5) I-700 low; T6) I-700 up and low; T7) I-700 + I-125 up; T8) I-700 + 125 low; T9) I-700 up and I-125 low; T10) I-125 up and I-700 low. Plants were sampled 1 month after germination and nodules from both strains recognized by their external colour. Results of T1, T2, T4 and T5 indicate that the location of the inoculum did not affect the total nodule dry wt. and the ureide content of nodules and petiole. In T3, 50% of the total nodule dry wt. and ureide content were detected at each nodulated root level, the total being equal to T1 or T2. However, in T6, 70% of the total nodule dry wt. and ureide content were found in the upper nodulated area of the roots, the total being equal to T4 or T5. In mixed inoculum (T7 and T8) the strain I-700 dominated over I-125 at the upper but not at the lower root position. Competition between strains for nodulation was only observed when both strains were placed together at 5 cm deep since in T9 and T10 each of the strains behaved as in T1, T2, T4 and T5. The leaf area, shoot and root dry wt. and ureide content of plants were not affected by the treatments.

D-18

Influence of soil conditions upon competitive nodulation.

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It has been observed that soil conditions can influence the outcome of competitive nodulation, yet the scientific reasons are not understood. Despite the identification of nine antigenically distinct groups of indigenous Rhizobium leguminosarum bv. trifolii in the Abiqua soil, 72% to 91% of the root nodules of subclover were consistently occupied by two serotypes, AS6 and AN21, when field grown plants were sampled for two consecutive years. Observations were made to suggest that unusual conditions existed at the time of nodulation in the field. Seedlings were observed to germinate in response to either evening dew or modest precipitation in early September, but did not nodulate until the return of more extensive precipitation in late October. Serotypes AS6 and AN21 occupied between 71% and 80% of nodules from both field germinated seedlings transferred to the laboratory prior to nodule development, and the nodules of seedlings allowed to nodulate under field conditions. In contrast, neither AS6 nor AN21 dominated nodule occupancy to the same extent when subclover was grown in potted soil regardless of the temperature of root growth or the moisture conditions under which the soil was equilibrated prior to planting. However, when field soil was placed in direct contact with seedlings on agar slants, nodule occupancy was dominated by AS6 and AN21.

D-19

Serological relationships between Rhizobium leguminosarum bvs. trifolii and viceae in soil. S. Strain* and P.J. Bottomley. Dept. of Microbiology, Oregon State University, Corvallis, OR 97331-3804, USA.

Subterranean clover (Trifolium subterraneum) nodulates with both Rhizobium leguminosarum bvs. trifolii and viceae. A major concern in our ecological studies was the possibility that fluorescent antibodies raised to serotypes of bv. trifolii might cross-react with members of the soil population of bv. viceae. Eighty-five isolates of bv. viceae were recovered from root nodules of Pisum sativum and three Vicia spp. grown in subclover pasture soil. Ninety-two percent of these isolates cross-reacted with four of the nine antisera previously raised to bv. trifolii isolates recovered from the same soil. This finding was confirmed in a replicate experiment with soil collected from the same field site and also when nodules were sampled from native Vicia plants collected from a nearby unimproved hillside. Serological analysis indicated that 80% to 83% of the isolates cross-reacted with four antisera. These findings confirm those of Young and co-workers, which were based upon multilocus enzyme electrophoresis, that soil populations of bvs. trifolii and viceae are related. Despite the same serotypes being confirmed within the populations of both biovars, their population densities in soil were dramatically different. In the subclover pasture bvs. viceae and trifolii were 10^2 and 10^6 g^{-1}, respectively, whereas reciprocal densities were measured in the soil from the unimproved hillside where Vicia spp. were the dominant legume cover and where Trifolium spp. were virtually absent.

D-20

Enumeration and immunohistrochemical identification of rhizobial strains in soybean nodules by ELISA. T. Sato*, Y. Nohara[1] and S. Sugarawa. Dept. of Agric. Chem. Obihiro Univ. of Agric. and Vet. Med. Obihiro, Hokkaido 080, Japan. [1]Serotec Co. Ltd. Ebetsu, Hokkaido 069, Japan.

In this study ELISA, more quantitative than fluorescent antibody method, were applied to compare infectivity of 3 soybean rhizobial strains (OUG 115, OUG 117 and S 32) and to observe nodule sections immunohistochemically.
Surface sterilized soybean seeds were sown, inoculated with a mixture of an equal amount of 3 strains, and grown in a growth chamber for 45 days. Strains in nodules were analyzed by ELISA. Ratio of singly infected nodules with OUG 115, OUG 117 and S 32 were 3, 9 and 47%, respectively, when inoculum size was 10^6 cells. About 40% nodules were doubly or triply infected ones, and in those S 32 was more competitive than the other. With increasing size of inoculum S 32 gave increasing infectivity. Enzyme linked immunohistochemical method was applied for observation of doubly infected nodules (OUG 115 and OUG 117,. After treatment with peroxidase labeled anti 115 Ab (P-115 Ab), infected cells by OUG 115 exhibited brown color with diaminobenzidine. When the adjacent section was treated with P-117 Ab, the infected cells, which did not develop color with P-115 Ab, exhibited red color with 3-amino-9-ethylcarbazole.

D-21

Screening for bacteriocin-producing strains among collections of *Rhizobium meliloti* and *Bradyrhizobium japonicum*. Brenda T. Breil* and Eric W. Triplett. Dept. of Agronomy and the Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, Madison, WI 53706-1597, USA

Strains of *R. meliloti* and *B. japonicum* in the collections of the USDA and Lipha Chemical Co. were screened for bacteriocin production on minimal media using a plate assay.

Of 195 *R. meliloti* strains tested at pH 6.8, seven strains were found which inhibited more than 30% of the *R. meliloti* strains. One strain, 102L1, inhibited 70% of all of the strains in the collections and inhibited 81% of those strains isolated from East, Southeast, and Midwest regions of the United States. One strain of *R. meliloti*, 102F69, is very inhibitory toward 95% of the strains of *R. leguminosarum* bvs. *phaseoli*, *viceae*, and *trifolii* in our collection but has no effect on most strains of *R. fredii* and *R. meliloti*. Another strain, 102F61, inhibted 100% of *R.l. viceae*, 63% of *R.l.* bv. *phaseoli*, 39% of *R. meliloti*, 33% of *R. fredii*, and none of the *R.l.* bv. *trifolii* strains tested.

Three hundred and ninety four strains of *B. japonicum* were tested for bacteriocin production against *B. japonicum* USDA strains 110, 123, 127, 129, and 138 at roughly pH 6.1. The five strains which most strongly inhibited the test strains were chosen for further study. All five bacteriocin-producing strains are Nod^+ and Fix^+ and preliminary results suggest that inhibition by these strains is usually greater on minimal as opposed to enriched media. The effect of pH on bacteriocin production by these five strains and their ability to inhibit bacteria other than bradyrhizobia will be presented.

D-22

Further characterization of the trifolitoxin production and resistance genes (*tfx*) from *Rhizobium leguminosarum* bv. *trifolii* T24. Malgre Carreno* and Eric Triplett. Dept. of Agronomy and the Center for the Study of Nitrogen fixation. University of Wisconsin-Madison, Madison, WI. 53706, USA.

Trifolitoxin, produced by *Rhizobium leguminosarum* bv. *trifolii* T24, is a bacteriostatic anti-rhizobial peptide. Previous studies from this laboratory have identified a 4.4 kb (kilobase) region, referred to as *tfx*, which encodes for both trifolitoxin production and resistance. In the present work, recombinant plasmids containing the *tfx* region, pTFX1 (50 kb) and pTFX2 (23.7 kb), were digested with *Mlu* I, *Spe* I, and *Eco* RI. Restriction fragments which contained all or most of the *tfx* region were isolated by electroelution. These fragments were then singly or doubly digested with several restriction enzymes and analyzed by gel electrophoresis. With these data a detailed restriction map of *tfx* is proposed.

The localization of restriction sites within *tfx*, and its flanking sides are necessary in order to sequence by means of the ExoMeth sequencing method (J.A. Sorge and L.A. Blinderman. 1989. Proc. Natl. Acad. Sci. [USA] 86: 9208-9212). This sequencing approach does not require subcloning provided that 5' overhangs or blunt ends and 3' overhangs can be generated on either end outside the region of interest. *Mlu* I and *Spe* I digests provide the necessary 5' overhangs on either side of *tfx* while *Nsi* I and *Kpn* I generate 3' overhangs within *tfx*. Other restriction enzymes such as *Aat* II, *Sac* I, and *Sfi* I generate 3' overhangs outside *tfx*.

D-23 Ecotoxicological Assessment of Heavy Metals with Melanin Producing (Mel+Fix+) Rhizobium Strains.

Minocher Reporter*, Wanda Baune (NSI Technology Services Corp.), & Lawrence Kapustka, U.S. EPA, Environmental Research Laboratory, Corvallis, OR 97333.

Melanin production in three strains of Rhizobium was studied. These three strains, R. meliloti, R. fredii, and R. leguminosarum bv. phaseoli, were obtained from J. Beringer (see Cubo et al., Appl. & Environ. Microbiol. 54, 1812-1817, 1988). Melanin was released from these strains within three hours by SDS in the presence of tyrosine and copper. We found these strains capable of melanin production when copper was substituted by cadmium, lead, or zinc. Controls did not produce melanin. R. meliloti strain GR015 gave the most consistent melanin response to metal exposure. Melanin production and growth in culture are being evaluated in the development of a biological method for the assessment of heavy metals. Companion tests using acetylene reduction and melanin production by nodulated plants, using filter-based assays are also being studied since one mel gene of each Rhizobium strain is located in the respective symbiotic plasmid (Cubo et al., 1988) and is a regulatory gene which may be linked to the regulatory nif A gene in certain symbiotic bacteria (Borthakur et al., Mol. Gen. Genet. 207, 155-160, 1987). Melanin is, however, not required for nitrogen fixation (Borthakur et al., 1987). Melanin production has not been detected in Bradyrhizobium by Cubo et al. (1988).

D-24 Heavy metal toxicity and tolerance in *Rhizobium leguminosarum* bv. *trifolii*.

Ken Giller*, Amar Chaudri and Steve McGrath[2], Wye College, University of London, Wye, Ashford, Kent TN25 5AH. UK. [2]Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ. UK.

N_2-fixation and yield of clover (*Trifolium repens* L.) were reduced drastically in field plots where the soil is contaminated with heavy metals at concentrations close to those permitted under current guidelines for environmental protection. (McGrath *et al.*, 1988). The failure of N_2-fixation in metal-contaminated soil was due to the survival of just a single, ineffective *Rhizobium* strain (Giller *et al.*, 1989). If effective strains of *Rhizobium* were inoculated into metal-contaminated soil (10^7 cells/ g soil) none survived after two months of incubation. Survival tests of these strains in dilute solutions of Zn, Cu and Cd have shown differences in tolerance to heavy metal concentrations. The ineffective strain isolated from metal-contaminated soil is tolerant to greater concentrations of Zn and Cd whilst there is little difference in tolerance to Cu. This probably explains the ability of the ineffective strain to survive in the soil which is contaminated with Zn, Cd and Cu as Cu is much less available in soil. We have now studied survival of *Rhizobium leguminosarum* bv. *trifolii* in many UK soils and find that there are generally no strains which can survive in soils contaminated with heavy metals.

McGrath *et al.*, (1988) Soil Biol. Biochem. 20. 415-424.
Giller *et al.*, (1989 Soil Biol. Biochem. 21. 841-848.

D-25

The immediate fate of N_2 fixed by *Gloeothece* growing under alternating light and darkness. J.R. Gallon*, S.M. Perry and K.J. Flynn, Biochemistry Research Group, School of Biological Sciences, University College of Swansea, Singleton Park, Swansea, SA2 8PP, UK.

In cultures of *Gloeothece* growing under alternating light and darkness, most N_2 fixation occurs during the dark period. In contrast, with the notable exception of nitrogenase itself, most protein synthesis occurs during the period of illumination. Newly fixed nitrogen is not, therefore, immediately used for bulk protein synthesis.

During the dark period, there was no accumulation of intracellular free amino acids in *Gloeothece*, except for a slight increase in arginine. Similarly, there were only small changes in the intracellular concentrations of nitrogen reserves such as phycobiliproteins and cyanophycin. On the other hand, there was a marked accumulation of extracellular glycine, serine and alanine. Furthermore, these amino acids disappeared from the growth medium during the light period.

It is therefore proposed that *Gloeothece* releases most newly fixed nitrogen as simple amino acids into the extensive slime capsule that surrounds this cyanobacterium. Subsequently, these amino acids reenter the *Gloeothece* cells in order to support protein synthesis in the light.

D-26

"Incomplete Symbiosis" of Azolla

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"Incomplete Symbiosis" was found in Azolla inoculated with cultured Anabaena which was isolated by Newton and Bai. Only some of the Anabaena filaments were observed to enter leaf cavities. Many stayed outside the leaf cavity and formed connection from leaf to leaf. Azolla with "incomplete symbiosis" did fix N_2(ARA) but its N_2-fixing capacity was not enough to satisfy the nitrogen amount needed for growth. Most plants failed to survive in N-free medium but multiplied normally in N-containing culture solution. Incomplete Symbiosis may be dependent on the biology of Anabaena inocula used. This new finding opened a new way for the study on the symbiotic relationship between Anabaena and Azolla.

D-27 Some characteristics of plasmids in Rhizobia isolated from Leucaena leucocephala.

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Rhizobia symbiotic with Leucaena leucocephala have been considered as the fast grower, but the occurence of plasmids has not been reported up until now. Using a mild procedure to isolate large cccDNA, the presence of one or several plasmids in these rhizobial cells were exactly demonstrated in our experiment. The plasmids were detected by agarose gel electrophoresis and the molecular weight estimated in comparison with Rhizobium fredii strain USDA191. All the rhizobial strains from L. leucocephala, TAL1145, CB81, MXLL1, MXLL2, MXLL3, MXLL6 and MXLL7, contain one or several plasmids. The molecular weight of these plasmids in 95Md-195Md. We also found that IncP1 group plasmid, RP1:Tn501 occurred in Pseudomonas aeruginosa strain PA09501, can be transferred into strain TAL1145 and from TAL1145 into E. coli strain HB101. The DNA-DNA hybrization experiment showed the plasmids of strains MXLL2 and MXLL7 carrying nod and nif genes.

D-28 Rhizobium population genetics: diversity of plasmid profiles in Sinorhizobium fredii strains isolated from soybean cultivar Aijiaozao.

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Sinorhizobium fredii strains were isolated from soybean (Glycine max L. Merr.) cultivar Aijiaozao grown in a soil planted with soybean for at leart two decades without inoculation in central China. All nodules were collected from each sampled plant, and one rhizobial isolate was obtained from each nodule. Ninety-three fast-growing soybean rhizobium isolates were examined for plasmid content by a modified Eckhardt gel protocol. One to five plasmids were reproducibly detected in the isolates. The sizes of plasmids ranged from approximately 30MD to 500MD. According to the size and number of plasmids, it was possible to classify the 93 isolates into 22 different groups. The dominant group contained 16% of the isolates. The isolates in the dominant group had only one plasmid. This diversity of plasmid profiles demonstrates the existence of a great heterogeneity of plasmid pools within newly isolated fast-growing soybean rhizobia.

D-29

A study on the rhizobiophages of soybean. Z.F.Wang*, H.Xu and Y.H.Con.Wuhan Institute of Virology.Chinese Academy of Sciences.Wuhan,430071.P.R.China.

A number of soybean rhizobiophage strains infective to Bradyrhizobium japonicum or Rhizobium fredii were isolated from soybean-growth soils in China.Seven among the rhizobiophages obtained were characterized by their morphology, host range, restriction endonuclease patterns (REP),G+C % and DNA-DNA homology.It was shown by electromicroscopy that all phages isolated could be differentiated into four morphotypes. Various morphotypes were different in REP. The molecular weights of the rhizobiophages were 40.0 and 70.7 kb. The G+C% content of the DNAs ranged between 41. 5 and 72.1 mol %(Tm). All the seven phage strains had narrow host ranges within 14 rhizobial strains tested. But DNA-DNA hybridization between them showed homology to a certain extent. Five out of the seven phage strains. i. e. psx, psc, pfm, pfc and pfx had the capacity of generalized transduction. The transdution frequencies were 1×10^{-4} to 1×10^{-7} per recipient.

D-30

Characterization of Rhizobium trifolii bacteriophage isolated from Iraqi Soil. F.Y.AL-Ani*, A.K.AL-Sultani and I.A.M.-Khafagi, Genetic engineering and biotechnology research centre. Baghdad , IRAQ.

Rhizobium trifolii bacteriophage (RT5) was isolated from soil near Baghdad . The morphological feature of RT5 seems to be a Bradly type A structure with a hexagonal head (86 nm length) and long contractile tail (101 nm length). The plaques of the phage were clear and rounded with diameter ranging from 1-4 mm. RT5 had a latent period of 110 min with arise time of 90 min and average burst size of 100. The host range of the phage appeared to be limited since it lysed single Rhizobium strain out of eighteen indicator strains. Antiphage serum was prepared in rabbit in order to determine its specificity. Results indicate that neutralization was complete in case antiserum-phage mixture while no inactivation of phage was detected in control. Thermostability studies showed that the phage remained stable at 40C for 1hr. while 94% of the phage particles were inactivated after heating for 1hr at 50C . Moreover the phage was stable between PH5-10. RT5 was found to be unaffected by chloroform and diethyl ether. Experiments with Mitomycin C, indicates that the nucleic acid of RT5 is DNA.

D-31

Symbiotic efficiency of two *Rhizobium meliloti* isolates of the same phage type with different denitrification activities. Y-K. Chan. Plant Research Centre, Agriculture Canada, C.E.F., Ottawa, Ontario, K1A 0C6, Canada.

Isolates 123 and 250 of *R. meliloti* are of the same phage type, with the same plasmid profile and originate from the same cultivation site. Isolate 123 was found to reduce nitrate faster than isolate 250 while the latter showed a higher nitrite reduction activity in denitrifying culture. The effect of nitrate on their symbiotic efficiency with *Medicago sativa* cv. Saranac was compared 3 weeks after inoculation. Treatment with nitrate up to 10 mM for 1 to 7 days had no significant effect on plant dry weight or nodule growth in all cases. Acetylene reduction by plants nodulated by isolate 250 was inhibited by nitrate at 5 mM. However, the activity of those nodulated by isolate 123 was not inhibited by 5 or 10 mM nitrate. In this case, the simultaneous nitrous oxide production as an indicator of denitrification was consistently higher than that by plants nodulated by isolate 250. It appears, therefore, that the tolerance of whole-plant nitrogen fixation to nitrate is related to denitrification activity.

D-32

Effects of nitrate on Carbon and Nitrogen metabolism in Lupinus albus nodules along the growth cycle. M.A. Chamber* and A. Serrano, D.G.I.E.A.,S.I.A., Apdo. Oficial, Alcalá del Río, 41200-Sevilla, SPAIN.

Two strains of Bradyrhizobium sp. (Lupinus), namely 750 and IM 438, previously characterized as possessing different types of nitrate-reduction and hydrogen-uptake activities, were inoculated on Lupinus albus seedlings planted in Leonard jars under 0 and 8 mM $N\text{-}NO_3^-$. Some parameters related to the symbiosis were determined in 6 controls along the period of growth. The strain, level of combined N and date of sampling significantly influenced the fresh weights, protein contents and Relative Efficiencies of nodules; H_2-uptake and nitrite production by bacteroids varied at the same level of probability by all of the three factors studied. Otherwise, numbers, carbon contents and nitrogenase activities of nodules, and total N in shoots displayed significant F by nitrate. Contrarily, C/N in nodules and dry matter accumulation in plants did not change by either the strain inoculated or the concentration of nitrate in the medium, only the symbiosis stage seemed to affect them. Nitrate-reduction and H_2-uptake in bacteroids were significantly correlated (R= -0.49).

D-33 **Leaf sampling strategies for ^{15}N-dilution studies of N-fixing legume tree species under field conditions.**
D.D. Baker*, Yale University, School of Forestry and Environmental Studies, New Haven, CT USA and M. S. R. Rao, Central Soil and Water Conservation Research and Training Institute, Bellary, INDIA.

An initial labelling/sampling experiment was conducted to determine the problems of applying the ^{15}N-dilution method to nitrogen-fixing tree species under field conditions. A plantation of three legume tree species (Enterolobium cyclocarpum, Leucaena hybrid, and Periserianthes falcataria) were each interplanted with Eucalyptus grandis at a field site in Hawaii. The 7.5 x 10 meter blocks were isolated from the surrounding soil by a plastic barrier placed in a border trench. ^{15}N-labelled ammonium sulfate was applied to the soil within the trenched block three months after the seedlings were planted. At nine months leaf samples were taken from trees within the blocks using two sampling strategies. The leaves were dried, ground and analyzed for ^{15}N content. A statistical analysis of the data will be presented to describe the difficulties of applying dilution studies to tree species and to suggest possible solutions to these difficulties.

D-34 The effect of root exudates on the growth and activity of nitrogen-fixing bacteria in the rhizosphere of pearl millet. S. L. Albrecht* and J. R. Milam. USDA-ARS, Agronomy Dept. & Dept. of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611 USA.

Nitrogen-fixing bacteria in the rhizosphere may make significant contributions to the nitrogen economy of many plants. Pearl miliet breeding lines, which support different levels of nitrogenase activity in their rhizosphere, were grown in a greenhouse, labeled with ^{14}C and the amount of the label in root exudates was determined. Rhizosphere nitrogenase activity and the populations of inoculated nitrogen-fixing bacteria were also monitored. The populations of nitrogen-fixing bacteria and associated nitrogenase activity increased during the course of the experiment, however, the specific nitrogenase activity remained relatively constant. The root exudates had no measurable deleterious affect on either growth or nitrogenase activity of the bacteria when grown in pure culture. There were positive correlations between nitrogenase activity and the loss of recent photosynthate by the roots and the population of nitrogen fixing-bacteria in the plant rhizosphere and the loss of recent photosynthate by the roots. It was concluded that, under the conditions of this experiment, the mechanism that allows certain breeding lines to support greater amounts of nitrogen fixation was quantity of root exudate provided to the nitrogen-fixing bacteria.

D-35

Root growth of *Vicia faba* in relation to its nodulation by *Rhizobium leguminosarum*. P. Roakothan*, Institute of Plant Agronomy and Plant Breeding, University of Gottingen, D-3400 Gottingen, Germany F. R.

Seventeen European *Vicia faba* varieties were inoculated with 6 *Rhizobium leguminosarum* strains characterized in symbiosis in earlier experiments with faba beans by 126C13 Hup+ (nod$^+$ fix$^-$), 14(1) (nod$^+$ fix$^+$), Hoh Erb2 (nod$^-$), 3853 Hup$^+$ (nod$^-$/nod$^-$ fix$^+$/nod$^+$ fix$^+$). Five replications of each combination were grown under greenhouse conditions by using a root sterile plant culture method. Plants were harvested after 12 weeks. Productivity and nodulation characters such as dry matter of shoot and root as well as number and weight of nodules were recorded.

Table 1: Absolute and relative dry matter production of the shoot (root=1) in relation to the inoculated *Rhizobium* strains

Rhizobium strain	n	mean* (relation)	Mean*[dry matter root (in g)]
Hoh Erb2	133	1,1	1,233
128C13 Hup$^+$	134	1,1	1,27
3853 Hup$^+$	148	1,7	0,895
14(1)	139	2,3	1,035

*Differences significant at p = 0.01, with the exception of that between Hoh Erb2 and 128C13 Hup$^+$

Absolute as well as relative dry matter production of the roots of those plants inoculated with ineffective strains (nod$^-$, nod$^+$ fix$^-$) were increased compared to those inoculated with effective strains (nod$^+$ Fix$^+$). Therefore, it is imaginable that e.g., in the case of plant nodulation with strains of high symbiotic performance the root architecture is so changed, that under water stress conditions the growth of faba beans may be limited by the root system established under optimum conditions during the early growth and development of the plant.

D-36

Xanthobacter as a rhizosphere organism of rice. H.K. Reding*, P.G. Hartel and J. Wiegel. Dept. of Microbiology, The University of Georgia, Athens, GA 30602, U.S.A.

The genus *Xanthobacter* is composed of gram-type negative, pleomorphic bacteria capable of fixing dinitrogen while growing chemolithotrophically. Isolates of *Xanthobacter* have been obtained from marine and freshwater sediments, sewage samples, street ditches and soil of meadows. In one study, several strains were isolated from the rhizosphere of rice in Japan; however, *Xanthobacter's* role as a rhizosphere organism was not further investigated. Samples from the soil and rhizosphere from a rice field in Southeast, Arkansas, U.S.A. yielded 1.38 x 10^4 *Xanthobacter*/ g dry soil and 7.8 x 10^5/ g dry root (rhizosphere/ soil = 57). After successive washings of the rice roots for 1 min, 5 min, and 10 min using a stomacher blender, 2.2 x 10^3 *Xanthobacter*/ g root remained attached to the root. In studies using rhizosphere assemblies, *Xanthobacter* isolate KR-JW1 was able to bind to the root hairs and root surface and also multiply in the rice rhizosphere with numbers increasing from 10^5 CFU/mg root to $10^{7.5}$ CFU/mg root in one day while remaining at that concentration for the remainder of the study (7 days). Plants inoculated with 10^5 CFU *Xanthobacter* and cultivated gnotobiotically for 14 days had shoot dry weights 40% higher than the uninoculated controls.

D-37 Some Characteristics of *Rhizobium leguminosarum* Isolates from Uninoculated Field-grown Lentils.

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The ICARDA lentil growing region includes a wide diversity of soils, climates, lentil management and history of lentil production. Indigenous populations of *Rhizobium leguminosarum* were sampled from nodules of lentil (*Lens culinaris* Med) growing in 7 countries from ICARDA region.

Enumeration of this *Rhizobium* using most probable number (MPN) showed that 70% of the sampled sites possessed < 10^4 cells/g of soil.

The symbiotic effectiveness of all isolates was measured in aseptic hydroponic gravel culture system. The proportion of highly effective isolates was greater among those collected from Turkey and Jordan amounting up to 50% of the total isolates, whereas the moderately and low N_2 fixing isolates were the predominant in Syria, Egypt and north west African areas. In salinity and heat tolerant studies, only 21.8% of the isolates could grow on medium with 0.5% NaCl, whereas 2.6% tolerated up to 1% NaCl. Four percent of the isolates could grow at 40°C.

The degree of similarity between the isolates from and within different regions was studied using intrinsic antibiotic resistance (IAR) against 8 antibiotics in 20 concentrations. The highest similarity was among isolates from Jordan and Turkey (79 and 62%) and the lowest among isolates from other regions (>35%)

D-38 The occurrence of nodulation in Brazil, and nodule structure of legume species.

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This paper summarises the nodulation surveys in the South East, North East and North of Brazil in the latest 5 years. Observation were on 621 legume species, 318 of which are species not previously reported about their nodulation status, including 35 new genera, (thirteen nodulating and twenty two non nodulating ones). Anatomical studies have been carried out on nodules from different tribes of the Leguminosae together with the rhizobial root infection. As a result,a new root nodule classification based on how the host plant respond to the bacterial invasion developing the nodule structure is emerged. Four different ways of nodule development are recognised; exclusively via persistent infection threads, exclusively via non persistent infection threads, infection threads together with mitosis of the infected cells and exclusively via mitosis of the infected cells. The distribution on these types of nodules in the Leguminosae, the mophology and the evolutionary relationships will be discussed.

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D-39 Distribution and density of VA Mycorrhizae and *Rhizobium leguminosarum* Biovar *Viciae* associated with Faba bean growth in major Egyptian soils

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Faba bean plants and soil samples were collected from different locations in Egypt. Soil samples represent calcareous soil, sandy soil, salt affected soil, alkaline soil and alluvial clayloam soil. Roots of Faba bean plants were screened for the VA mycorrhizal infection and nodulation by specific *Rhizobium*. Numbers of mycorrhizal spores and *R.leguminosarum* biovar *Viciae* cells were enumerated in different soil samples. Physical and chemical properties of soil samples were also determined. It was found that the development of *R.leguminosarum* biovar *Viciae* generally increase VAM fungal colonization and spores production. The number of nodules per plant and the weight of nodule mass were significantly correlated with level of mycorrhizal infection.

D-40 Geographic distribution and environmental conditions that affect the presence and abundance of *Rhizobium leguminosarum* biovar *phaseoli* in a tropical area.

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Ninety-four samples were collected in a tropical area (Costa Rica) covering eight different rainfall ranges (<1500-6500 mm/year) and twelve different soil types, to determine the effect of different environmental conditions on the distribution and number of the indigenous population of *Rhizobium leguminosarum* biovar *phaseoli*. Climatic data (rainfall and temperature), soil characteristics, presence of legumes, and total number of soil microorganisms were correlated with abundance of rhizobium at each different site using a principle components technique. *Rhizobium* was widely distributed and present at all the sites with the exception of four and, in general, higher numbers of bacteria were found in sites where the host (*Phaseolus vulgaris* L.) was present.

D-41 Nodulation study of soybean under Iraqi conditions.

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Inoculation was increased seed yield, number of pods/plant, seed weight, protein content,while decreased the percentage of shriveled seed and oil content. Nodule formed only on soybean plants received inoculum. Generally, low N levels resulted in more nodules/plant than high N levels. Increasing N levels tended to decrease fresh and dry weight of nodules. Inoculated plants were taller than uninoculated plants. Increasing N levels from zero to 160 kg/ha had increased seed yield, number of pods/plant, seed weight and decreased the percentage of shriveled seed.

D-42 Ontogenetic changes in natural ^{15}N abundance in the tissues of stem-nodulating legumes and estimates of N_2 fixation by these plants.

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Two stem-nodulating legumes (Sesbania rostrata and Aschynomene indica) were grown during summer seasons in upland and paddy fields. The accumulation of N and variations of natural ^{15}N abundances ($\delta\ ^{15}N$) in plant parts were investigated. Two nonnodulating soybeans grown under upland conditions and three weeds harvested from paddy fields were used as the reference plants.

Under both soil conditions the two stem-nodulating species grew well with nodules on stem and/or roots. The $\delta\ ^{15}N$ values of the leaves plus stems and fruits of nonnodulating plants were elevated while those of stem-nodulating plants were slightly negative (from -0.7 to -0.1‰). However, the $\delta\ ^{15}N$ values of stem and root nodules of S. rostrata and A. indica were high (from +6 to +11‰). The $\delta\ ^{15}N$ values of the bacteroid fractions in stem nodules as well as in root nodules from these two stem-nodulating plants were higher (from +8 to +15‰) than those of the cytosol and cortical fractions. The analysis of xylem fluids and nodule tissues of S. rostrata and A. indica indicated amides as the major form of N transport.

The percent N derived from N_2 fixation in the tops of S. rostrata and A. indica were 65 and almost 100% in upland fields and 78 and 93% in paddy fields, respectively. The amounts of fixed N in S. rostrata and A. indica at the final harvest were 15 to 28 g and 8 to 13 g per m^2, respectively.

D-43

Metabolic and structural condition of *Frankia* in root nodules of *Alnus incana* exposed to prolonged darkening.
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Symbiotic *Frankia* is dependent on photosynthetic assimilates from the host plant for the supply of energy. This flow can be interrupted by prolonged darkening of the plants. Cuttings of *Alnus incana* (L.) Moench in symbiosis with a local source of *Frankia* were exposed to darkening for 2-4 days under controlled climate conditions. At various times *Frankia* vesicle clusters were prepared from the root nodules. Saturating amounts of four different substrates for respiration were supplied to the vesicle clusters and the respiratory capacity was used as a measure of the condition of *Frankia*. Nitrogenase activity in intact plants and in the vesicle cluster preparations were also followed.
During darkness the respiratory capacity of *Frankia* decreased, and was almost lost after four days in darkness. The recovery was studied by ending the dark treatment after two days and restoring the usual day/night cycle. Mechanisms for the effect of prolonged darkening on *Frankia* activity are discussed in relation to structural observations in transmission electron microscopy.

D-44

Stimulation of Hippophae growth by Frankia inoculation. Oleg S.Stupar*, Svetlana N.Novik. Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino on the Oka, 142292, USSR

Inoculation of actinorhizal plants by Frankia under sterile conditions greatly increases their growth. The data on field inoculation, i.e. in the presence of a natural pool of Frankia, are much more scarce, especially for Hippophae.
We present the results of laboratory and field inoculation of Hippophae rhamnoides L. by Frankia sp. VKM 1096D and 1097D, isolated from Shepherdia argentea Nutt.
Inoculation in a 3-month greenhouse trial on sterile soil (Perlite, peat-sand mixture) increases the shoot length 2.7 times and weight of plants up to 4.3 times as much as that of non-inoculated plants.
2-year trials on experimental plots of coal mine spoils in South Ukraine and fruit gardens at the Middle Volga have shown that inoculation increases the viability of seedlings (up to 60%) as well as the shoot length (20-25%), weight of plants (1.6 times) and root nodules (8.8 times as much).
These data suggest that Frankia inoculation can be used as an agricultural method to stimulate viability and growth of Hippophae rhamnoides L.

D-45 Detecting *Frankia* in soil using PCR. D.D. Myrold*, A.B. Hilger, and S.H. Strauss. Dept. of Soil Science and Dept. of Forest Science, Oregon State University, Corvallis, OR 97331-2213, USA.

Frankia are present in low numbers in most soils, at least as determined using a plant bioassay method. Consequently, to detect *Frankia* in soil and follow its population dynamics, it was necessary to develop a technique that was capable of detecting only a few *Frankia* per gram of soil. We have adapted a direct lysis procedure for extracting DNA from soil that works well even in soils with high amounts of humic substances. Basically, this extraction procedure involves a lysozyme digest, lysis with SDS and proteinase K, purification by potassium acetate precipitation, ethanol precipition to concentrate the DNA, and agarose gel electrophoresis to remove humic contaminants. This method yielded approximately 10% of DNA from added *Frankia* cells; the DNA obtained was pure enough for use in RFLP analyses and for PCR amplification. The primers used for PCR amplification flank a 381-bp sequence of the *nif*H gene. We used a booster PCR procedure to enhance the fidelity and increase the sensitivity of PCR amplification. Using this method we were able to detect less than 1 pg (about 100 copies of the *nif*H gene) of *Frankia* DNA against a background of 1 μg of total DNA.

D-46 **SURVEY OF *FRANKIA* ASSOCIATION IN NONLEGUMINOUS TREES OF NORTH-EASTERN INDIA.** ***Jha, D.K., Sharma, G.D. and Mishra, R.R.** Department of Botany, N.E.H.U., Shillong. India.

Trees growing under different forest ecosystems on the foothills of North-Eastern region of India were investigated for their actinorhizal association. Out of ten plant species studied, three had nitrogen fixing actinorhizal nodules. These included *Alnus nepalensis*, *Myrica esculenta* and *Eleaegnus latifolia*. *Frankia* was isolated from the root nodules of *A. nepalensis* using different defined mediums. The nitrogenase activity of root nodules as well as *Frankia* isolates were measured. Morphology of nodules varied from host to host. The nitrogen fixing ability of nodules was related to specificity of *Frankia*.

D-47 *In Vitro* Sporulation of *Frankia*. S.H. Burleigh and J.O. Dawson*. 1301 West Gregory Dr., Dept. of Forestry, Univ. of Illinois, Urbana, IL, USA, 61801

Sporulation is a common attribute of *Frankia* strains and has a profound effect on the actinorhizal symbiosis. Sporulation within nodules of actinorhizal plants is associated with decreased nitrogen fixation and *Frankia* spores greatly increase the nodulation potential of inoculum for actinorhizal plants. We have studied *in vitro* sporulation of *Frankia* strains CcI3, ArI3, and CpI1. Nitrogen and phosphate inhibited *in vitro* sporulation in these strains. Ammonium-deficient medium increased *in vitro* sporulation of CcI3 from zero to 200,000 spores per ml and Ammonium- and phosphate-deficient medium increased sporulation from zero to 6,000,000 spores per ml of nutrient medium. The effects of temperature, aeration and solution osmolarity on *in vitro* sporulation of *Frankia* will also be discussed.

D-48 Heat and cold shock proteins in arctic and temperate strains of rhizobia. J. Cloutier*, D. Prévost and P. Nadeau, Agriculture Canada, Sainte-Foy, Québec, Canada G1V 2J3, and H. Antoun, Dép. des Sols, Université Laval, Québec, Canada G1K 7P4

The exposure of bacteria to temperature above normal for growth induces heat shock proteins (hsp). Only few studies reported cold shock protein (csp) synthesis in bacterial cells exposed to low temperature. Arctic rhizobia, isolated from *Astragalus* and *Oxytropis* spp., have shown cold adaptation for growth and nitrogenase activity. The purpose of the present study was to compare the hsp and csp induced in 3 arctic (psychrophilic) and 3 temperate (mesophilic) *Rhizobium* strains in order to identify some of the mechanisms involved in thermoadaptation. Bacterial cells were grown at their optimal temperatures (arctic 25°C, temperate 30°C). Log phase cells were pulse-labelled with ^{35}S-methionine and exposed to different shock temperatures selected in order to compare proteins of all strains at a given temperature and at a given ΔT ($T°$ shock - $T°$ optimal). Analysis by electrophoresis and fluorography revealed that three hsp of high M.W. (107, 83, 59.5 kD) were present in all strains when exposed to 2°C over their maximal growth temperature (arctic 30°C, temperate 36.4°C) and at 41.4°C. They were also present in arctic strains exposed at 36.4°C. At 46.4°C, there was no hsp induced in temperate strains (ΔT = 16.4°C), whereas they were present in all arctic strains (ΔT = 21.4°C). This first report on hsp on rhizobia shows that cold adapted arctic rhizobia can synthesize hsp at temperatures higher than temperate rhizobia. Preliminary results on csp revealed that strains used in this study can also synthesize proteins at temperatures below their minimum growth temperature.

D-49

Interaction between subarctic populations of white clover and *Rhizobium*. M. M. Svenning* and B. Solheim, Dept. of Plant Physiology and Microbiology, IBG, University of Tromsø, N-9000 Tromsø, Norway.

Symbiosis between white clover (*Trifolium repens* L.) collected at different latitudes in Norway and *Rhizobium leguminosarum* biovar *trifolii* populations from the same localities was assessed. The evaluation was done with plants grown in Leonard jars. Uninoculated nitrogen fertilized plants were used as controls. The results showed a statistically significant interaction between the host and symbiont populations. White clover from the most northern latitudes produced more plant material when nodulated by bacteria from the north than from the south. At first harvest dry weight of the plant materials from the least and most effective symbiotic combinations were 20% and 50% of their respective controls. But at second harvest it was 40% and 90% of their controls.

D-50

Effect of water potential and temperature on carbon and nitrogen fixation by some subaerial cyanobacteria. S.N.Tripathi* and E.R.S.Talpasayi, Department of Botany, Banaras Hindu University,Varanasi-221005, INDIA.

In tropics, rain-fed building surfaces exposed to sun are found predominantly colonized with non-spore forming firm and coloured sheath containing cyanobacterial species of Gloeocapsa, Scytonema and Tolypothrix which are exposed to a high degree of variability in humidity (20-85% RH), temperature (6-44 ^{o}C), and light intensity (250-436 cal/cm^{2} /day) and are often exposed to temperature of 60-70 ^{o}C for a few hours at midday in summer. Filamentous crust comprised of Scytonema and Tolypothrix, when subjected to various water potentials and temperatures, showed optimum ^{14}C-fixation and acetylene reduction at 0 bar water potential and 30 ^{o}C temperature. Further, acetylene reduction was found much sensitive to lowering in water potential compared to ^{14}C-fixation by the algal crust. A decrease of 45,65 and 90% in ^{14}C-fixation by the crust was observed on lowering of osmotic water potential to -14, -28 and -46 bar, respectively, while it was 72% decrease in acetylene reduction even at -14 bar osmotic water potential. When dry crusts exposed to higher temperature (55 to 110 ^{o}C) for one hour were grown, they did not show much variation in ^{14}C-fixation and acetylene reduction, compared to that of unexposed.

D-51

Nitrogenase, nitrate reductase and photosynthetic activities of black alder exposed to cold temperatures. Christoph S. Vogel* and Jeffrey O. Dawson. Department of Forestry, University of Illinois, 1301 W. Gregory Dr., Urbana, IL 61801.

Nodulated black alders (Alnus glutinosa [L.] Gaertn.) receiving NH_4NO_3 fertilization exhibit net decreases of up to 38% in foliar N concentration during autumn while non-nodulated alders receiving the same N fertilization show no decrease in foliar N concentration. Exposure to 1.5 hours of 4, 1 and -1^{o} C temperatures decreased nodular acetylene reduction rates by an average of 46% in NO_3^- fertilized black alders. Partial to full recovery of acetylene reduction activity occurred within 7 days after cold treatments. Nitrate reductase activities of roots and leaves were not significantly affected by cold treatments. Photosynthetic rates declined by an average of 17% after cold treatments and recovered partially after 8 days. These results are consistent with the idea that alder net foliar N resorption during autumn is greatest in alders that derive the greatest proportion of their nitrogen from symbiotic nitrogen fixation, which is impaired more readily by low temperatures than is nitrate reductase activity.

D-52

Federal regulation of *Rhizobium* and *Bradyrhizobium*: USDA-APHIS' view. S. Shantharam, USDA-APHIS/BBEP, Hyattsville, MD 20782, U. S. A.

At present, the Animal and Plant health Inspection Service (APHIS), a regulatory branch of the U. S. Department of Agriculture (USDA) has listed *Rhizobium* and *Bradyrhizobium* under the list of regulated organisms under 7 CFR 340 entitled Plant Pests; Introduction of Genetically Engineered Organisms or Products, Final Rule of June 16, 1987. The rationale used was to include the lowest possible taxonomic unit that may be or may contain plant pests, and are regulated if they meet the definition of plant pest. The plant pest is defined as "any organism belonging to any taxa contained within any listed genera or taxa is only considered to be a plant pest if the organism can directly or indirectly injure, or cause disease, or damage in any plants or parts thereof, or any processed , manufactured, or other products of plants." As such, when some strains of *Bradyrhizobium* that nodulate soybeans were found to cause chlorotic symptoms albeit transiently, such strains were deemed plant pathogens. However, in practice, APHIS has never required a permit for planned release of genetically engineered *Rhizobium* and *Bradyrhizobium*, following individual scientific review. APHIS has issued several opinion letters where it has categorically mentioned that the species and strains of bacteria belonging to these two genera are not plant pests and as such a permit is not required.

The agency has now undertaken the task of reviewing all the available scientific data and has sought a survey of opinions from the scientific community to review the plant pest status of *Rhizobium* and *Bradyrhizobium*. Some of the results of the survey and the available scientific data will be presented for discussion.

D-53

RISK EVALUATION FOR GENETICALLY MODIFIED MICROBIAL INOCULANTS

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[2] Dipartimento di Biotecnologie Agrarie, Universita di Padova, Via Gradenigo 6, 35131 Padova (Italy)

This work is aimed at developing appropriate biological material and tools to monitor survival, persistence and competition of Rhizobium, Bradyrhizobium and fluorescent Pseudomonas strains in the soil and rhizosphere and to evaluate potential gene transfer unambiguously and on a routine basis.

Construction of appropriate, genetically modified, inoculant strains involved the exploitation of the lacZ gene as a suitable marker system. Fluorescent Pseudomonas do not possess lactose catabolic genes and in Bradyrhizobium and R. leguminosarum the lacZ gene is only expressed at a low level. In order to distinguish between the lactose marker and any resident or fortuitously acquired lactose gene, the reporter gene was (a) combined with the inducible dct promoter that responds to a known environmental signal other than lactose and (b) fused to a highly expressed synthetic δ^{70} consensus promoter. To ensure stable maintenance of the dct-lacZ fusion, this marker gene was integrated into the genome without disrupting cellular functions in Rhizobium. Introduction of Hg-resistance genes into R. leguminosarum allowed easier recovery of introduced strains present at low densities in soil and rhizosphere. Vectors are being developed in which the antibiotic resistance markers are replaced by the ThyA gene. This gene provides a positively selectable marker for the introduction and retention of plasmids in host cells which are thymidylate-synthase negative.

D-54

Population Behavior of Genetically-Engineered *B. japonicum* in an Open-Air Soybean Field Trial. *T.R. Hankinson, P.E. Sweeney*, S.M Gudbrandsen, and C.W. Ronson. Biotechnica International, Inc., 85 Bolton St., Cambridge, MA, 02140*

On June 16, 1989, Biotechnica International, Inc. initiated a soybean field trial in Baton Rouge, Louisiana, in collaboration with Louisiana State University. The field trial was designed to determine whether a specific genetic modification in *Bradyrhizobium japonicum* shown to increase foliar yield in the greenhouse would also increase bean yield in the field. The behavior of two genetically-engineered derivatives of a member of serogroup 110 used as seed inocula was closely monitored throughout the field season and compared to the behavior of the 110 wild type strain.

The three inocula developed similar population profiles in the inner rhizosphere, establishing a population of approximately 1 x 10^5 cells *per* gram dry soil by Day 9 and increasing lognormally with time to approximately 1.6 x 10^8 cells *per* gram dry soil by Day 120. This response by *B. japonicum* to the soybean rhizosphere, and the response elicited by *Rhizobium meliloti* inocula in the alfalfa rhizosphere (*see accompanying abstract*), are distinctly different. Populations in the outer rhizosphere also increased lognormally with time. Lateral migration six inches from inoculated plant rows was never detected, nor was vertical migration to a depth of 12 inches. Nodule occupancy ranged from 21% to 72%, with the majority of inoculant nodules occurring in the crown region of the plant root. Additional information regarding post-harvest population behavior will also be presented.

SESSION E

Bacterial systems
E-01 - E-110

E-01

Genomic Analysis of Rhizobiaceae: Size Estimation, Sequence Analysis, and Analysis of Rare-cutting Restriction Endonucleases. Bruno W. S. Sobral, Rhonda J. Honeycutt, and Alan G. Atherly*. Department of Genetics, Iowa State University, Ames, Iowa 50011.

We prepared intact genomic DNA from Rhizobiaceae in agarose plugs and used pulsed-field gel electrophoresis (PFGE) to compare the genomes of free-living and bacteroid forms of bradyrhizobia, and to investigate the stability of whole genomic fingerprints produced by rare-cutting enzymes. No differences were observed between free-living and two developmental stages of bacteroid forms of *B. japonicum*, when analyzed by field inversion gel electrophoresis (FIGE). Southern analysis was used to localize *nod* and *nif* genes to DNA fragments produced by digestion with rare-cutting restriction endonucleases. *B. japonicum* USDA 123 genomic fingerprints were stable after passage through nodules and after maintenance on rich growth media for up to 100 generations. These data suggest that major DNA rearrangements do not occur during bacteroid development, after bacteroid dedifferentiation, or by maintenance on rich growth media. The genome size of *R. meliloti* and *B. japonicum* was estimated as 4.7 and 5.7 megabases (Mb), respectively. Finally, the results of our work will be useful for the application of PFG techniques to evolutionary studies and physical mapping of Rhizobiaceae bacteria.

E-02

Studies on the characterization of *R. meliloti nodD3* gene. G.Q. Yu and J.B. Zhu*. Laboratory of Molecular Genetics, Shanghai Institute of Plant Physiology, Academia Sinica. Shanghai 200032, CHINA

Three *nodD* gene copies are involved in the regulation of *nod* gene expression. The genes *nodD1* and *nodD2* are plant factor-luteolin dependent, whlie *nodD3* is luteolin independent in activating the structural *nod* genes. Based upon the sequencing data of the *nodD3*, we found a notable diversity occuring in the C-terminal domain of the *nodD3* proteins. Furthermore, we also demonstrated the characteristic structure of *nodD3* promoter which differs markedly from the *nodD1* and *nodD2* promoters.

Experiments using T7 RNA polymerase/promoter system for testing the expression of *nodD3* gene in *E. coli*, 3 different sized polypeptides 35Kd, 37Kd and 43Kd were generated from the cloned *nodD3*. Our preliminary result suggests that only one of these three polypeptides functions in activating the expression of *nod* genes without luteolin.

E-03

Characterization of *Rhizobium meliloti* mutant T37 with delayed nodulation and decreased competitiveness.
B.V.SIMAROV, O.P.ONISHCHUK, L.A.SHARYPOVA, O.N.CHESNOKOVA. All-Union Research Institute for Agricultural Microbiology, Leningrad, 189620, USSR.

R.meliloti mutant T37 with delayed nodulation ($Noi^{\pm}$) was isolated by the use of Tn5-mutagenesis in strain CXM1-105. T37 had the same symbiotic efficiency(Eff^{++}) as parental strain, but characterized with low competitiveness ($Comp^{-}$). In order to study connection between nodulation and competitiveness kanamycin resistance was transduced by the use of phage øM12 from T37 to CXM1-105. 15 transductants were analysed in microvegetation experiments. All of them inherited $Noi^{\pm}$ and $Comp^{-}$ phenotypes. These results allow to suppose that nodulation is one of the factors determining nodulation competitiveness. In order to localize Tn5-insertion in genome of T37 kanamycine resistance was transfered to avirulent *R.meliloti* mutants ZB121 and CXM1-219 by the use of plasmid RP4-4. All recombinants obtained were able to induce nitrogen-fixing nodules on alfalfa. This demonstrates that Tn5 had inserted into *Sym*-plasmid. Eckhardt gel electrophoresis of plasmid confirmed the transfer of *Sym*-plasmid. $Noi^{\pm}$ mutation in T37 was not complemented by the largest R-prime pGR1, carrying *nod* and *hsn* genes R.meliloti. This indicates that in mutant T37 Tn5 labels a new locus on *Sym*-plasmid, which controls both nodulation and competitiveness.

E-04

Localization of mutations enhancing symbiotic efficiency on *Rhizobium meliloti* megaplasmids.
L.A.SHARYPOVA, J.G.FOMINA-ESHCHENKO, B.V.SIMAROV. All-Union Research Institute for Agricultural Microbiology, Leningrad, 189620, USSR.

R.meliloti has two large plasmids with the same electrophoretic mobility: M1, controling nodulation (Nod) and M2, controling EPS synthesis (Exo). By the use of Tn5-mutagenesis we have isolated 19 *R.meliloti* mutants with enhanced symbiotic efficiency - abiliti to increase plant mass (Eff^{n}). Southern blot analisis revealed that 9 mutants had Tn5 insertions in one of megaplasmids. Eff^{n} mutants were conjugated to *R.meliloti* mutant ZB121 and *Agrobacterium tumefaciens* mutant UBAPF2, using mobilizing system pSUP102::Tn5. Mutant ZB121 (Exo^{-},Nod^{-}) carried 3 plasmids: a-cryptic one (140 MD), b-deletion derevative of M1, c-M2. Such plasmid content allowed to identify easily what megaplasmid was transferred to ZB121. In the case of M1 transfer from 4 Eff^{n} mutants recombinants lost b-plasmid (on the basis of plasmid incompatibiliti), preserved Exo^{-}-phenotype of the recipient and acquired Nod^{+}-phenotype of the donor. *A.tumefaciens* recombinants from the same mutants acquired Nod^{+}-phenotype too. In the case of M2 transfer from 5 Eff^{n} mutants recombinants had the same plasmid pattern as ZB121 but obtained Exo^{+}-phenotype. *A.tumefaciens* recombinants retained Nod^{-}-phenotype. Results obtained demonstrate that both megaplasmids of *R.meliloti* take part in control of symbiotic efficiency.

E-05

Generation of medium-size self-transmissible plasmids harbouring nod genes in Rhizobium meliloti.

Rumayuntseva M.L., Provorov N.A., Simarov B.V.
All-Union Research Institute for Agricultural Microbiology, Leningrad, 189620, USSR.

114 independent Tn5-mob recombinants obtained in Nod^+Fix^+ R. meliloti strain 135a were crossed with Nod^- mutant CXM1-46 using RP4-4. Donor strain harbours 2 megaplasmids with Mm 120 MD (which is devoid of nod genes) while recipient has 2 megaplasmids only. In 18 crosses Nod^+Fix^+ transconjugants were obtained with frequency of 10^{-5} - 10^{-6} per recipient after selection for $Km^rSm^rRif^s$ phenotype (donor was markered by Rif^r, recipient - by Sm^r). In all transconjgants a plasmid of nearly 60 MD was detected by Eckhardt procedure. Transfer of 3 of these plasmids from CXM1-46 transconjugants , which were Tc^s and didn't harbour RP4-4, to Agrobacterium tumefaciens GM1-9023 resulted in Nod^+Fix^- phenotype. Frequency of recombinants was 10^{-8} per recipient. Spontaneous elimination of transferred plasmids from A. tumefaciens recombinants caused the loss of virulence. Southern hybridization with pKSK5 revealed presence of "common" nod genes in these plasmids. Possible mechanisms of generation of the medium-size self-transmissible nodulation plasmids in R. meliloti are discussed.

E-06

Isolation and Characterization of Three Amino Acid Transaminase Genes from *Rhizobium meliloti.*

James R. Alfano* and Michael L. Kahn. Institute of Biological Chemistry and Department of Microbiology, Washington State University, Pullman, WA 99164-6340, USA.

Rhizobium meliloti genes encoding amino acid transaminase enzymes have been cloned by complementing an *E. coli* auxotroph using a *R. meliloti* gene library. A partial Sau3A digest of *R. meliloti* chromosomal DNA was cloned into the BamH1 site of pUC18. This gene library was then transformed into *E.coli* DL39, a strain that contains mutations in aspartate aminotransferase (*aspC*), aromatic transaminase (*tyrB*) and branched chain transaminase (*ilvE*). Transformants were placed on minimal media in the absence of specific amino acids. Distinct plasmids were isolated that relieved aspartate auxotrophy (pJA10 and, to some extent, pJA30), leucine auxotrophy (pJA20) and tyrosine and phenylalanine auxotrophy (pJA30). Extracts of DL39 have low transaminase activity. However, when oxaloacetate is used as the amino acceptor, DL39(pJA10) has high aspartate aminotransferase (AAT) activity and DL39(pJA30) has high aromatic transaminase activity and some AAT activity. pJA10 thus appears to carry the *R. meliloti aspC* gene and pJA30 the *R. meliloti tyrB* gene. The transaminase gene on pJA30 was insertionally inactivated with Tn5, and this mutation was then recombined into *R. meliloti* genome. The resulting transaminase mutant was Fix+. We are constructing *R. meliloti* mutants that lack the aspartate aminotransferase gene in pJA10 and the branched chain amino acid transaminase gene in pJA20.

E-07

Rhizobium meliloti and *Rhizobium leguminosarum dctD* Gene Products Bind to Tandem Sites in an Activation Sequence Located Upstream of σ^{54}-dependent *dctA* Promoters.

H. Ledebur, B. Gu, J. Sojda and B.T. Nixon*, Dept of Molecular and Cell Biology, Penn State University, University Park, PA 16802, USA.

Free-living rhizobia transport external C4-dicarboxylates to use as sole carbon sources and uptake of these compounds is essential for nitrogen fixation by rhizobial bacteroids. In both *Rhizobium leguminosarum* and *Rhizobium meliloti* the genes *dctB* and *dctD* are believed to form an *ntrB/ntrC*-like two component system which regulates the synthesis of a C4-dicarboxylate transport protein encoded by *dctA* (see Abstract by Gu and Nixon). In a recent comparison of the sequence of DNA between *dctA* and *dctB* genes of the two species we identified a partial dyad symmetry that is repeated upstream of putative *dctA* consensus promoters. We have now characterized some of the features of the *R. meliloti* and *R. leguminosarum dctA* promoters. Primer extension analysis of RNA isolated from free-living cells confirmed that initiation of *dctA* transcription likely occurs approximately 10 bases downstream of the previously identified σ^{54}-consensus sequences. By constructing chimeric *dctA-nifH* promoters and fusions of *dctA* to *lacZ*, we obtained evidence that full regulation of the initiation of transcription of *dctA* requires an additional upstream activation sequence. This UAS can function with either *dctA* or *nifH* σ^{54}-dependent promoters and reside at least 280 basepairs upstream of the site of transcription initiation. A DCTD-dependent binding activity, most likely the DCTD protein itself, was shown to interact with tandem sites in this upstream activation element. We conclude that, in both species, upstream DCTD binding sites are major determinants of the sensitivity of the *dctA* σ^{54}-promoter for the presence of external C4-dicarboxylates. Our data clearly indicate that fully regulated transcription of rhizobial *dctA* genes depends upon DCTD binding to an upstream repeat, but may suggest that some specificity is inherent to the promoter itself. The results confirm that σ^{54}-dependent promoters like *dctA*, that are now known to be widely distributed among procaryotes, may serve as model systems for understanding at least one mechanism of transcriptional activation by factors that bind at a distance. Further attempts to understand the more subtle interrelationships between σ^{54}-dependent systems like *nif, ntr* and *dct* may reveal how seemingly unrelated pathways are integrated into larger metabolic networks.

E-08

Rhizobium meliloti dctD Gene Product (DCTD) Has Three Functional Domains for Activating *dctA* Gene Expression.

B. Gu* and B.T. Nixon, Dept of Molecular and Cell Biology, Penn State University, University Park, PA 16802, USA.

Rhizobium meliloti dctA, dctB and *dctD* genes have been identified and sequenced. The genes *dctB* and *dctD* are thought to form a two-component system which responds to the presence of C_4-dicarboxylates to regulate the expression of a transport protein encoded by *dctA*. Homology with *Rhizobium leguminosarum* DCTD and other σ^{54}-dependent transcriptional regulators suggests that DCTD possesses three functional domains. We obtained evidence to confirm the existence of these domains by construction of various deletions of *R. meliloti dctD*, which were then expressed in both *E. coli* and *R. meliloti* in the presence of various reporter genes. Our results show that the DCTD central domain is sufficient to activate *dctA* gene expression. The DCTD amino-terminal domain represses this central domain activity, which can be released in *R. meliloti* in the presence of C_4-dicarboxylates. The C-terminal domain of DCTD can bind to an activation sequence located upstream of the *dctA* promoter (see Abstract by Ledebur et. al.), thus enhancing the ability of the central domain to promote transcription of *dctA*. In *E. coli*, the DCTD central domain can also activate expression of *R. meliloti* and *Bradyrhizobium* sp. [*Parasponiae*] *nifH* genes. In these assays, the *nifH* upstream activation elements were also functional, provided that the DCTD C-terminal domain was intact.

E-09

Modification of Expression of the Transcriptional Activator NifA in *Rhizobium meliloti* Enhances Symbiotic Nitrogen Fixation. *M.K. Williams*, J.L. Beynon, C.W. Ronson, and F.C. Cannon†. *BioTechnica International, Inc., 85 Bolton Street, Cambridge, MA, 02140. †Massachusetts General Hospital, Department of Molecular Biology, Boston, MA, 02144.*

We have investigated whether it is possible to genetically modify *Rhizobium meliloti* to obtain strains that improve alfalfa productivity through increased symbiotic nitrogen fixation. Based on several lines of evidence, we hypothesized that the level or timing of expression of NifA, the transcriptional activator of *nif* genes, may be a factor limiting nitrogen fixation. By modifying the expression of NifA, it may be possible to increase the expression of *nif* genes and achieve nitrogenase enzyme levels required to give enhanced nitrogen fixation.

To test this hypothesis, constitutive, heterologous and homologous promoters were fused to the *R. meliloti nifA* gene and the fusion genes were integrated into the inositol locus of the *R. meliloti* genome. The nitrogen-fixing abilities of the resultant strains were assayed by determining their effects on the biomass of inoculated plants grown under nitrogen-limiting conditions in the greenhouse. One strain, RMB7103, containing a particular fusion gene, gave a 3–7% increase over the wild-type control RCR2011 in the plant assays. A derivative strain, RMB7135, was constructed by introducing a second copy of the gene cassette into RMB7103 into another region of the *R. meliloti* genome. RMB7135 gave a 7–15% increase in alfalfa biomass when assayed in greenhouse conditions against wild-type strain RCR2011. The increase was statistically significant in each of three plant biomass experiments that were performed. Field trials of these genetically modified *R. meliloti* strains will be carried out in Wisconsin in 1990, pending EPA approval.

E-10

Isolation and characterization of oxygen-tolerant Rhizobium meliloti nifA mutants. R.Krey*, L.Sharypova, W.Klipp, and A.Pühler. Lehrstuhl für Genetik, Universität Bielefeld, Postfach 8640, D-4800 Bielefeld 1, FRG.

In R.meliloti the regulatory nifA gene is part of the nifA-nifB-fdxN transcription unit. Activation of nif promoters by R.meliloti NifA is oxygen-sensitive. To analyse this oxygen-sensitivity we developed a method for rapid identification of oxygen-tolerant nifA mutants. Point mutations in plasmids containing a constitutively expressed R.meliloti nifA gene were generated in vitro by hydroxylamine treatment. Oxygen-tolerant nifA mutants were identified by their ability to activate an R.meliloti nifH-lacZ fusion in Escherichia coli under high oxygen concentrations. We isolated a set of R.meliloti nifA mutants with increased oxygen-tolerance. On the basis of the specific NifA activity under different oxygen concentrations, these mutants could be divided into several classes. The oxygen-tolerant mutants were further cheracterized dy DNA sequence analysis. One mutant (Gly→Glu) maps within the interdomain linker region which is specific for oxygen-sensitive nifA gene products. Another mutant, which is located in the 3'-part of the central domain, did not result in a change of amino acid sequence. However, the mutant triplet shows a higher codon usage probability in E.coli.

E-11

Expression of R. meliloti nifA in different Rhizobium species.

C.L. Wang*. G.Q. Yu. J.B. Zhu and S.C. Shen. Laboratory of Molecular Genetics, Shanghai Institute of Plant Physiology, Academia Sinica. Shanghai 200032, CHINA

We have previously reported that R. meliloti nifA failed to express in R. trifolii and R. astragalus. To investigate whether Rhizobium nifA is host specific, the constructed Rm nifA-lacZ fusions were introduced into broad host range of cowpea Rhizobium R.sp280 for testing their activities. In these hybrids, Rm nifA-lacZ fusions were not inducible at all in microaerobic induction. When R. meliloti symbiotic megaplasmid marked with Tn5 was introduced into these hybrids, the transconjugants we found were able to form ineffective nodules on alfalfa, but did not show β-galactosidase activity.

In another experiment, when Rm P1-lacZ and Rm P2-lacZ fusions were introduced into cowpea Rhizobium R.sp280, β-galactosidase were not induced in microaerobic conditions or at symbiotic status of the rhizobia contained in the ineffective alfalfa nodules. However, when R.sp280 harboring these fusions were inoculating the plant siratro, very high level of β-galactosidase was demonstrated in the nodule bacteroids, indicating the activation of Rm P1 and Rm P2 by R.sp280 nifA under the influence of the host plant siratro.

E-12

IDENTIFICATION OF DOWNSTREAM ACTIVATOR SEQUENCES (DAS) ON THE NIFHDK PROMOTER OF RHIZOBIUM MELILOTI WHICH ARE ESSENTIAL FOR MICROAEROBIC BUT NOT FOR SYMBIOTIC EXPRESSION.

F. O'Gara*, B. Boesten, Y. Wang, K. Birkenhead and A. Dobson, National Food Biotechnology Centre and Microbiology Department, University College Cork, Ireland.

We are interested in investigating sequences on the nifHDK promoter which are involved in the regulation of this promoter under both microaerobic and symbiotic growth conditions. Oligonucleotide directed site mutagenesis has been used to construct deletions on the P1 promoter. These deletions which were constructed in a nifH::lacZ reporter plasmid were assayed for expression under microaerobic and symbiotic conditions.

Deletion of a 45 base pair region located between the promoter and the ribosome binding site [+15 to +59] results in a loss of expression of the P1 fusion under microaerobic conditions. However, a loss of expression was not observed under symbiotic conditions.

These results indicate that while this 45 base pair region is not essential for the symbiotic expression of a plasmid located nifH::lacZ fusion, it does contain sequences involved in the regulation of expression of this promoter under microaerobic conditions. The physiological significance of this region is under investigation.

E-13

Ferredoxin-like gene products from *Rhizobium meliloti* and their role in symbiotic nitrogen fixation.

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R.meliloti contains a number of genes necessary for symbiotic nitrogen fixation which do not have counterparts in *Klebsiella pneumoniae*. Two of these *fix* genes, *fdxN* and *fixX*, encode small ferredoxin-like proteins. A mutant in *R.meliloti fdxN* exhibits a Fix^- phenotype in contrast to similar mutants in *Azotobacter vinelandii* and *Bradyrhizobium japonicum*. The Fix^- phenotype of the *R.meliloti fdxN* mutant can be complemented by the *Rhizobium leguminosarum fdxN* gene, whereas no complementation could be observed for *nif*-associated genes encoding ferredoxins from *Azotobacter chroococcum* and *Rhodobacter capsulatus*. The role of FdxN in symbiotic nitrogen fixation was analyzed in an *in vitro* system. FdxN seems not to be involved in electron transport to nitrogenase but it is necessary for active nitrogenase component II. For biochemical analysis the *fdxN* gene product was overproduced in *Escherichia coli* and *R.meliloti* by use of expression vectors. For mutational analysis of *R.meliloti fixX* we constructed a defined non-polar mutation in *fixX*, which should not affect readthrough into *nifA*. This deletion mutant turned out to be Fix^-, indicating that *fixX* is also absolutely required for symbiotic nitrogen fixation.

E-14

Genetic studies of glutamate metabolism in *Rhizobium meliloti*.

J. L. Botsford,* Thomas Lewis, and Rigoberto Gonzalez. New Mexico State University. Las Cruces, New Mexico 88003, USA.

We have cloned the gene for glutamate synthase, *glt*. Using site directed mutagenesis and gene replacement, mutants auxotrophic for glutamate have been isolated. All mutants grow very slowly on minimal medium supplemented with glutamate and are unable to utilize amino acids as a source of assimilated nitrogen (Ntr^-). These mutants, like the wild type, accumulate glutamate when osmotically stressed. All are Fix^+. Suppressor mutants which remain auxotrophic but grow rapidly on minimal medium with glutamate or other amino acids (Ntr^+) can be isolated. An insertion mutation adjacent to *glt* and resulting in a Fix^- Glt^+ phenotype has been isolated.

E-15 The glutamine synthetases of R.leguminosarum and their regulatory genes. R. Defez, M. Chiurazzi, G. Manco, E. Patriarca, A. Lamberti, A. Riccio, C. Lopes, G. Raucci, S. Moreno, R. Meza, G. Espin and M. Iaccarino*. International Institute of Genetics & Biophysics, via Marconi 10, 80125 Napoli, Italy. Unidad de Biologia Molecular y Biotecnologia Vegetal, UNAM, 2-246, Cuernavaca, Mexico.

We will report on three different genes of R.leguminosarum expressing glutamine synthetase activities, GSI, GSII and GSIII. We studied their regulation at different levels: transcriptional analysis, post-translational modifications and regulatory genes controlling their expression. The ntrBC genes are required for expression of glnII (the gene coding for GSII), of glnB (the gene coding for PII) and, possibly also for expression of GSIII. A ntrC-like mutant is described in the Abstract of Moreno et al. We will present the sequence of ntrBC and of glnII, a transcriptional analysis of glnII and a detailed analysis of the glnB promoter fused to a reporter gene. After purification of GSI GSII, GSIII and PII, we obtained specific antisera and used them for immunological detection of the specific proteins, both by immunoblot and on thin sections of nodules. We will present data on the molecular weight of GSII and the possible relationship of an octamer-tetramer conversion to a post-translational modification.

E-16 Field isolates belonging to different biovars of *Rhizobium leguminosarum* can share identical chromosomal background. G. Laguerre, S. Mazurier and N. Amarger*. INRA Microbiologie des Sols, 21034 Dijon, France.

Four sub-populations of *Rhizobium leguminosarum*, one of biovar *phaseoli*, one of biovar *trifolii* and two of biovar *viciae*, were isolated from root nodules of french beans, red clovers, peas and vetches grown in one field. One hundred and twenty isolates per sub-population were characterized by plasmid profiles and a representative sample of one third was further characterized by total DNA restriction-digest patterns and hybridization patterns with chromosomal and pSym DNA probes.

The comparison of the different patterns allowed to differenciate 4, 5, 8 and 10 different chromosomal backgrounds for pea, clover, bean and lentil isolates, respectively. One of these chromosomal backgrounds, which was the dominant one of biovar *viciae* and biovar *trifolii*, was identical for the three biovars. It was identified in three quarter of the pea isolates, two third of the clover isolates, half of the lentil isolates and one third of the bean isolates. Another chromosomal background was common to 22 % of the clover and 5 % of the bean isolates while a third one was found in 4 % of the bean and 1 % of the lentil isolates. These results indicate that rhizobia with identical chromosome can harbour different host specificity determinants and that a same chromosomal genotype can form the majority of nodules in different species of legumes. The results also suggest that the different biovars of *R. leguminosarum* have not evolved separatly but are more probably the results of recent exchange or modifications of plasmid born genes. They also confirm that biovar cannot be considered as a taxonomic entity.

E-17

Cloning and characterization of a gene for production of a symbiotically repressed outer membrane protein of Rhizobium leguminosarum biovar viciae. Ruud A. de Maagd*, and Ben J.J. Lugtenberg. Leiden University, Department of Plant Molecular Biology, Botanical Laboratory, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands.

The outer membrane of R. leguminosarum shows a large number of changes during the differentiation from the free-living form to the bacteroid state. The nature of these changes, their regulation, and their role in normal nodule development, are major aims of our research. Bacteroids of R. leguminosarum biovar viciae strain 248 lack a group of antigenically related outer membrane proteins with molecular weights from 35 to 46 kDa. It was postulated that this group consists of two major proteins and in addition, their derivatives containing peptidoglycan subunits. In order to study the functions and regulation of expression of these outer membrane proteins, we have cloned a gene responsible for production of the 35 kDa outer membrane antigen. Monoclonal antibody 38, recognizing a strain-specific, surface-exposed epitope on oligomers of this protein, was used in a colony blot assay to pick up a clone from a cosmid-bank of strain 248 DNA, expressed in another Rhizobium strain. The nucleotide sequence of the gene responsible for production of the 35 kDa outer membrane antigen is presented. Both subcloning in the promoter-probe vector pMP220 as well as insertion mutagenesis with the promoter-probe transposon Tn5-B20(lacZ)were used to study the regulation of expression of this antigen at the transcriptional level.

E-18

Exported proteins encoded by nod genes from Rhizobium leguminosarum biovar viciae. A. Economou, W.D.O. Hamilton, A.W.B. Johnston and J.A. Downie*

In R. leguminosarum bv. viciae, 13 nodulation (nod) genes have been identified; nodD, nodABCIJ, nodFEL, nodMNT and nodO. Two of these genes, nodO and nodT encode proteins that have characteristics of proteins exported by bacteria.

The predicted protein sequence of nodO indicated that it is homologous to a group of bacterial proteins (including haemolysin, leukotoxin and proteases) that are exported into the growth medium of Gram negative bacteria. The NodO protein was found to be present in the growth medium of strains induced for nod gene expression and N-terminal sequencing of the NodO protein indicated that it (like haemolysin) is exported by a mechanism that does not involve N-terminal processing. Since a strain lacking its symbiotic plasmid but carrying the cloned nodO gene exported NodO protein it appears that the genes required for the export of NodO are not on the symbiotic plasmid. Significantly the NodO protein was found to bind Ca^{2+} suggesting a role for Ca^{2+} mediated attachment of NodO protein to plant cells.

The predicted sequence of the nodT gene suggests that it has a signal peptide typical of proteins which are destined for the outer membrane.

E-19 The nodA gene is physically and transcriptionally separated from the nodBC genes in Rhizobium leguminosarum bv. phaseoli type I strain CE3. M. Vázquez*, A. Dávalos, A. de las Peñas, J. Miranda, O. Santana, F. Sánchez and C. Quinto. Plant Molecular Biology and Biotechnology Unit . Apdo. Postal 2-246. Cuernavaca, Mor., MEXICO.

The common nodulation genes nodA, B and C are highly conserved between different Rhizobium and Bradyrhizobium species, and are required for the nodulation of legumes and non legumes. In almost all Rhizobium species so far described, these three genes are cotranscribed with nodIJ as an operon (nodA BCIJ).
We have isolated a cosmid that contains a region of the symbiotic plasmid of R. leguminosarum bv. phaseoli type I strain CE3. This region is capable of inducing ineffective nodules in a pSym$^-$ CE3 derivative (CFN2001). After mutagenesis of this cosmid we determined two separated zones essential for nodulation. One of these regions contains the nodBC genes by genetic and sequence criteria. An homogenotization of a mutation in the other zone on a pSym$^+$ background causes a Nod$^-$ phenotype and preliminar sequence data indicates that the mutated sequence is nodA. Therefore, we conclude here that in this R. phaseoli type I strain, the functional nodA gene is separated from nodBC and is transcribed independently of the latter genes.
This research was partially supported by grants from EEC and from the US NAS/ NRC by means of a grant from the US Agency for International Development.

E-20 Amplification and deletion events in the symbiotic plasmid of Rhizobium phaseoli. D. Romero*, M.L. Girard, S. Brom, J.M. Martínez-Salazar, M. Flores, R. Palacios and G. Dávila. Departamento de Genética Molecular, Centro de Investigación sobre Fijación de Nitrógeno, UNAM., Cuernavaca, Mor., México.

We have determined the physical map of the symbiotic plasmid (pSym) of R. phaseoli CFN42. This plasmid contains two nifHDK operons in direct orientation, separated by 125 Kb. Within this region there is a third nifH gene, located in inverse orientation, as well as some nodulation genes. Other reiterated sequences were located in several regions of the physical map. This structure suggests that the pSym must be prone to several kinds of genomic rearrangements. We have devised a system, based on a dose-dependent kanamycin resistance determinant and the sacB gene of Bacillus subtilis, which allows the positive selection of amplification and deletion events. The rearrangements more frequently found (10^{-3}) were amplification events, which increase two to three times the copy number of the 125 Kb region. Deletion events that eliminate the 125 Kb region were also observed, albeit at a lower frequency (10^{-4}). These rearrangements are frequently generated by recombination between the two nifHDK operons. Rearrangements similar to these, as well as loss or alteration of other plasmids present in this strain, have been found without any kind of selection. These rearrangements may explain the instability of symbiotic characters frequently observed in inoculant strains, and might be relevant to strain's adaptative potential.

E-21

Situation of temperate phage of *R. leguminosarum* bv. *trifolii* in root nodule cells. S. Higashi*. T. Uchiumi and M. Abe. Dept. of Biology, Fac. of Science, Kagoshima Univ. Kagoshima 890, Japan.

A temperate phage (ϕU) belonged to *Podoviridae* morphologically and had about 40 kb genomic DNA[1]. Lysogenization with phage ϕU affected the nodulation ability (Nod) of wild-type *Rhizobium leguminosarum* bv. *trifolii* 4S. Nod^- strains of 4S:ϕUs (lysogenic strains of 4S) lost *Sym* plasmid (315 kb), although Nod^+ strains kept it. Southern hybridization revealed that phage ϕU DNA integrated into different chromosome site in Nod^+ and Nod^- strains. Moreover, these strains showed different polypeptide patterns on 2-D PAGE. These results exhibit phage integrated sites are affecting the *Sym* plasmid replication directly or indirectly.

Phage induction from both free-living and bacteroid in the nodule cells could be occurred with mitomycin C treatment. A possibility that this phage may become a useful tool for gene transfer on the rhizobial symbiosis is also discussed.

1) Uchiumi, T., Ono, Y., Abe, M. and Higashi, S. 1989. J. Gen. Microbiol. *135*, 3133-3141.

E-22

Expression of heat induced proteins in wild type and plasmid-cured derivatives of Rhizobium leguminosarum bv trifolii. Dipankar Sen*, Jose Ivo Baldani, and R.W. Weaver. Dept. of Soil and Crop Sciences, Texas A & M University, College Station, Texas 77843.

Inoculant rhizobia have not persisted well enough in soils in Texas to form nodules on reseeding clover. Plasmid profiles of rhizobia isolated from nodules of reseeding clover did not match with those of inoculant strains. Soils in Texas undergo numerous cycles of heating and drying during the year. We are investigating whether plasmids play a role in providing resistance to heat and drought stress. Protein profiles of a wild type isolate containing four plasmids and four of its derivatives with each of the plasmids cured were compared when grown in broth cultures at 25°C for 24 hours or when subjected to an elevated temperature of 40°C for the last four hours. Absence of the smallest(a) and the largest(d) plasmids failed to express a 27kD protein at 25°C or 40°C. Additionally absence of plasmid d did not express a 17kD protein at 25°C but expressed it at 40°C. At 40°C two proteins of mol. wt. close to 55kD had much enhanced expression in the wild type and cured derivatives except the one cured of plasmid b. The significance of these proteins and their relation to the plasmids is being investigated.

E-23

A *Rhizobium leguminosarum* protein homologous to *Escherichia coli* Fnr is involved in regulation of nitrogen fixation. S.Colonna-Romano, A.Schlüter, W. Arnold, A.Pühler and U.B.Priefer*. Lehrstuhl für Genetik, Universität Bielefeld, FRG

According to the current model, oxygen regulation of *nif* and *fix* genes in *R.meliloti* is promoted by a cascade regulatory circuit, involving the gene products encoded by *fixL*, *fixJ*, *fixK* and *nifA*. By interspecific complementation, a gene of *R.leguminosarum* bv. *viciae* was identified which is able to complement both, a *R.meliloti fixJ* and a *R.meliloti fixK* mutant in microaerobic (symbiotic) induction of the *nifA*-independent *fixN* gene cluster. Sequence analysis revealed that the corresponding gene product, designated FnrN, showed significant homology to *R.meliloti* FixK and to *E.coli* Fnr. The major difference between FnrN and FixK is the presence of 21 N-terminal amino acids in FnrN which include a putative metal binding cluster. A similar domain is also present in *E.coli* Fnr and is essential for oxygen dependent activity of the protein. It can therefore be assumed that, in *R.leguminosarum*, oxygen regulation of nitrogen fixation genes is mediated by FnrN. However, a *fnrN* mutant shows only a reduced nitrogenase activity, although the ability to induce the *R.meliloti fixN* gene is completely lost. The effect of FnrN on activation of *nif* and *fix* genes in *R.leguminosarum* is currently under investigation.

E-24

Molecular cloning of the *Rhizobium phaseoli recA* gene and construction of *recA* mutants. J.M. Martínez-Salazar*, D. Romero, M.L. Girard and G. Dávila. Departamento de Genética Molecular, Centro de Investigación sobre Fijación de Nitrógeno, UNAM., Cuernavaca, Mor., México.

The *R. phaseoli recA* gene was isolated from a genome bank constructed in lambda 1059 by interspecific complementation of the Fec- phenotype. A region carrying the *recA* gene was obtained by sucloning into PSUP202 and functional complementation of an *Escherichia coli recA* mutant. Approximate localization of the *recA* gene was determined by mutagenesis with MudIIPR13 and heterologous hybridization with an *E. coli* intra-*recA* probe. Complementation studies of *E. coli recA* mutant with the *R. phaseoli recA* gene cloned into pSUP202 restored the resistance to DNA-damaging agents as well as recombination proficiency in Hfr- mediated conjugation and P1-mediated transduction. The interposon Sp^r/Sm^r from pHP45 was inserted into the *recA* gene and introduced in two *R. phaseoli* strains by homogenotization. The *R. phaseoli recA* mutants were more sensitive to DNA-damaging agents and have a 100-fold reduction in the frequency of recombination.

E-25

A mutant of Rhizobium leguminosarum biovar phaseoli altered in the regulation of nitrogen metabolism. S. Moreno[1], M. Chiurazzi[2], R. Meza[1], R. Defez[2], M. Iaccarino[2] and G. Espín[1-2*]. [1]Plant Molecular Biology and Biotechnology Unit. UNAM. Apdo. Postal 2-246, Cuernavaca, Morelos, Mexico. [2]Istituto Internazionale di Genetica e Biofisica, CNR, via Marconi 10, 80125, Napoli, Italia.

A Tn5 insertion mutant, strain CFN2012, of Rhizobium leguminosarum biovar phaseoli devoid of glutamine synthetase II (GSII) activity was analysed. Strain CFN2012 is defective in glnB expression since P_{II}, its product, is not detectable. The level of adenylylation of GSI is impaired in strain CFN2012 suggesting a role for the P_{II} protein in the adenylylation mechanism. A DNA fragment was isolated that complements the GSII, PII and adenylylation defects and shows cross hybridization to the ntrC gene of Agrobacterium tumefaciens, suggesting that the phenotype of CFN2012 is due to a ntrC::Tn5 insertion. In contrast to ntrC mutants of other Rhizobiacea strain CFN2012 grows on nitrate.

E-26

Isolation of *Rhizobium galegae* strain-specific DNA probes by subtraction hybridization.

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A simplified version of mixed-phase subtraction hybridization (Bjourson and Cooper, 1988) was tested to isolate strain-specific DNA sequences for *Rhizobium galegae* identification. The subtracter DNAs (*R. galegae* HAMBI 1428 and HAMBI 1462, 25 µg of each) were denatured, mixed, and placed on nitrocellulose filter (Schleicher & Schuell BAS 85, 0,45 µm) and immobilized (80°C, vacuum, 2 h, or UV 254 nm, 30 sec.). The probe DNA (*R. g.* HAMBI 1174) was digested with MboI, denatured, and hybridized overnight with the subtracter DNAs on the filter. The hybridization solution was thereafter purified through a NENSORB 20 (DuPont) cartridge to collect the putative strain-specific DNA mixture. The specificity of the DNA was tested on dot-blots with a non-radioactive labeling and hybridization technique (Boehringer-Mannheim).

E-27

Expression of *Rhizobium galegae* common *nod* clones in various backgrounds.

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The cosmid clone pRg30, carrying common nodulation genes of *Rhizobium galegae* HAMBI 1174, and pRg33, a subclone of pRg30 containing a 5,7 kb ClaI insert carrying *nod*DABC, were conjugated into different backgrounds and used in expression analyses in plant tests.
The nodulation ability of the *nod*C mutant strain *R. meliloti* 1126 was restored to wild type level in alfalfa. Derivatives of *R. leguminosarum* strain 5039 carrying Tn5 inserts in *nod*A, B and C genes, complemented with pRg30 and pRg33, showed delayed nodulation in *Vicia sativa*. pRg30 conjugated into *Agrobacterium tumefaciens* C58C1 and tested on *Galega orientalis* plants produced root hair deformation and tumour like structures.

E-28

Cloning of the host specific gene region of *Rhizobium galegae*

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More than 2000 colonies from the clone bank of *Rhizobium galegae* HAMBI 1174 were assayed for the presence of the 25 bp *nod*-box sequence.
Total DNA of *R.galegae* and plasmid DNAs from nine different clones showing strong homology with labeled *nod*-box were digested with EcoR1, blotted on nitrocellulose and hybridized with the same probe.
Four EcoR1 fragments sized 9.2, 4.6, 3.9 and 3.3 kb were found to contain all *nod*-boxes of this strain. The 9.2 kb fragment carries common nodulation genes cloned in pRg30 and all other *nod*-box sequences are found in pRg10, the plasmid carrying putative genes for host specificity.
Rhizobium meliloti genes *nod*D1, D2, D3, *hsn*ABC and D, and *nif*HDK were used as probes when analysing the gene region adjacent to the *nod*-box sequences.

E-29

Indentification of *nod* and *nif* genes on pRaZ15 from *Rhizobium astragali* gene library. Zhang Zhongming, Zhou Junchu, Li Fudi and Chen Huakui, Biotechnology Cente of Huazhong Agr. University, Wuhan, 430070, P. R. China.

Total DNA partially digested by EcoRI was prepared from *R. astragali* 7653R. From which 20-30 kb fragments were collected by sucrose gradient centrifugation. They were then ligated to the EcoRI froagment of a broad host range cosmid pLAFR1. The ligated DNA was packaged in vitro by packaging mix prepared from E. coli 2688 and 2690. 7×10^4 Tc^r clones were obtained and 50 of them were chosen for plasmid pattern test. The frequency of recombinant clones was about 30% in the gene library. The size of cloned foreign DNA fragments varied from 18.8-29.8kb. A positive clone with *common nod* genes was identified by DNA hybridization with a 8.7kb *nod* fragment from pRmSL26. Tri-parental mating was conducted between gene library and a Nod^- psym-cured strain 7653R+1 with the help of pRK2013. Trans conjugats restored both of its nodulation and nitrogenase (detected by acedylene reduction method) activities on *Astragalus sinicus*. Isolates from nodules were proved to contain a plasmid, named pRaZ15. Fragments of pRaZ15 digested by Sal I or EcoR$_1$ were hybridized with the *nif* HDK prob pSA30. Transconjugats were also obtained by tri-parental mating between pRaZ15 and *R. leguminosarum* T83K3 or biovar Phaseolus 3622-15. Both of them could form nodules on *A. sinicus*. The results suggested that *common nod*, *hsn* and *nif* genes were located on pRaZ15 from *R. astragali* 7653R gene library.

E-30

Mutants of rhizobia with altered responses of symbiotic N_2 fixation to combined nitrogen. **K.Zlotnikov*, M.Kadyrova. Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, Moscow Region, 142292, USSR**

Inhibitory effect of combined nitrogen on nitrogenase activity of legume root nodules is well known (1). It is believed that the influence of nitrogen on symbiotic N_2 fixation is mediated exclusively by the host plant thus leaving for rhizobia a passive role. We describe mutants of *R.phaseoli* and *B.japonicum* which have acquired the capacity to influence the level of sensitivity of symbiotic N_2 fixation to combined nitrogen. Most such mutants were found in bacteria resistant to high levels of NH_4^+ or NO_3^- in rich agar media. Based on the studies of the mutants, we conclude that rhizobia play an active role in mediating the response of symbiotic N_2 fixation to combined nitrogen.

(1) Streeter, J. (1988). CRC Crit.Rev.Plant Sci. 7:1-24.

E-31

Expression of *Bradyrhizobium japonicum* Nodulation Gene In *Rhizobium fredii* Nod^- Mutants. Lu- Ming Zhou*, Ying-Hua Cen. Guangdong Institute of Microbiology, Guang-zhou, China.

The pLAFR1 gene library of *B. japonicum* USDA110 was transferred by the helper plasmid pRK2013 into Tn5 induced Nod^- mutants of *Rhizobium fredii* USDA 191-4. The Sm^R Tc^R transconjugants were ocurred with the frequency of 5×10^{-4}. After purification, these transconjugants were used en masse to inoculate germinated soybean seeds. Seven nodules were obtained from the nodulation experiment. The fast-growing Nod^+ Sm^R Tc^R *Rhizobium fredii* strain was isolated from each nodule. All these isolates gained a new plasmid with the molecular weight about 51Kb. This recombinant plasmid could be transferred by helper pRK2013 into *E.coli* HB101 with the frequency of 1×10^{-4}. Digested with EcoR1, all the recombinant plasmids produced a ~23Kb fragment and a ~6.5Kb fragment besides the vector fragment. Using a ^{32}P labeled nod gene probe prepared from pRmSL26, the DNA to DNA hybridization test confirmed that a~20kb EcoRI-BamHI DNA fragment from these plasmids showed sequence homology with nod gene probe from *R. meliloti*.

E-32

Mutations in the Rhizobitoxine Genes of *Bradyrhizobium japonicum* have no Affect on Nodule Formation with *Glycine max*. X. Ruan and N.K. Peters. Biotechnology Center and Dept. of Agronomy, the Ohio State University, Columbus, OH 43210-1002 USA.

Bradyrhizobium japonicum USDA 61 was mutated with Tn5. Two thousand nine hundred indiviuals carrying Tn5 were screened for the synthesis of rhizobitoxine. The screen was based on the inhibition of b-cystathionase by rhizobitoxine. Eleven individuals which did not produce rhizobitoxine were identified and characterized. Inoculation of *Glycine max* (Williams) and *Glycine soja* with these rhizobitoxine mutants resulted in normal nodule formation and nitrogen fixation. Further investigation is needed to explore the biological importance of rhizobitoxine.

E-33

Protoplast fusion in Rhizobium sp TAL 141 ure his and Rhizobium japonicum USDA 192 trp phe. P. Thipayathasana*, S. Sidhipraneed and S. Sukwatanasinit. Dept. of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

Rhizobium sp TAL 141 was characterized as a suitable donor strain for a stress-tolerant genetic reccombination in case, that an intra or an inter-reccombination event could be provided. A double mutant, identified as ure his was isolated by a twice successive NTG mutagenesis from the TAL 141 and was subjected for a furthur protoplast formation experiment. Under our performed condition, more than 90% of protoplast could be formed from the TAL 141 ure his strain. Rhizobium japonicum USDA 192 trp phe was a strain of a point mutation isolated from the parental USDA 192 being formerly characterized as a stress sensitive strain. Under an appropriate condition, 95% of protoplast was formed from the USDA 192 trp phe cells. The protoplast fusion between them was performed in the presence of 15% PEG at 42°C 5 min. Fusants were allowed o grow on our designed MMG plates. Some of the grown cells were selected and reisolated and purified. Five different phenotypic strains so-called, F_{11}, F_{14}, F_{15}, F_{20} and F_{36} were subjected for a further biochemical studies. In this communication, the physiological and the biochemical properties of the fusants will be illustrated in parallel to their original parental strains.

E-34

Two functional homologs of the σ^{54} gene (*rpoN*, *ntrA*) in *Bradyrhizobium japonicum*. H.M. Fischer*, I. Kullik, B. Herzog, H. Knobel, S. Fritsche, T. Bruderer and H. Hennecke. Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland.

Recognition of -24/-12-type promoters by RNA polymerase requires a specific sigma factor, σ^{54} (RpoN, NtrA). In the nitrogen fixing soybean symbiont, *Bradyrhizobium japonicum*, two separate, strongly conserved *rpoN* genes (78 % identical aa) were identified and sequenced. A comparison of RpoN1 (484 aa) with RpoN2 (537 aa) revealed the presence of an extra stretch of 48 aa in RpoN2 between the short N-terminal and the large C-terminal, highly conserved domains. Individual mutagenesis of *rpoN1* and *rpoN2* by gene disruption resulted in *B.japonicum* mutants which both were Nod^+Fix^+ on soybean whereas a *rpoN1/2* double mutant had a Nod^+Fix^- phenotype. This suggested that both *rpoN* copies were functional and could replace each other. The influence of *rpoN* mutations on gene expression from different *nif-* and *fix*-promoters is currently under investigation. Using chromosomally integrated, translational fusions of *lacZ* to *rpoN1* and *rpoN2* a low, apparently constitutive expression of both genes was detected under aerobic conditions. Under anaerobiosis *rpoN1* expression was increased sixfold whereas *rpoN2* expression was the same as under aerobiosis.

E-35

Structural and functional analysis of the *fixLJ* genes of *Bradyrhizobium japonicum*. D. Anthamatten* and H. Hennecke. Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland.

Using the *fixLJ* genes of *Rhizobium meliloti* as a probe (1) we identified and cloned *fixLJ* of *B.japonicum*. Sequence analysis revealed that both genes are probably organized in an operon and are highly homologous (~60%) to their counterparts in *R.meliloti*. A nonpolar deletion of *fixL* resulted in a 90% reduction of Fix activity. The same phenotype was found for a *fixJ* insertion mutant. In *R.meliloti fixLJ* functions as a sensor/regulator system for *nifA* gene expression. To examine a possible role of *fixLJ* in the expression of the regulatory *fixRnifA* operon in *B.japonicum*, plasmid-coded *fixR'-'lacZ* fusions were introduced into *fixL* and *fixJ* mutant backgrounds. We found that the aerobic expression of the *fixRnifA* genes was not dependent on the presence of FixL or FixJ. Furthermore FixJ (which is supposed to be a DNA-binding protein) did not bind to the *fixR*-upstream DNA region. The data suggest that *fixL* and *fixJ* are not involved in the aerobic expression of *fixRnifA*. Under anaerobic conditions, *fixRnifA* operon expression is enhanced five-fold (2); whether or not the *fixLJ* genes are involved in this process is currently under investigation.

(1) David et al. (1988), Cell **54**: 671-683
(2) Thöny et al. (1989), J.Bacteriol. **171**: 4162-4169

E-36

A divergent *nod* box sequence is essential for *nodD1* induction in *B. japonicum*. Shui-ping Wang* and Gary Stacey. Dept. of Microbiology and Center for Legume Research, University of Tennessee, Knoxville, TN 37996.

Induction of *nod* genes in *Rhizobium* and *Bradyrhizobium* species is dependent on the presence of plant-produced flavonoids, the NodD protein, and a cis-acting *nod* box sequence, the binding site for NodD. *B. japonicum* is unique in that the *nodD1* gene is inducible by isoflavones in a similar manner to that of the *nodYABC* operon. A consensus *nod* box sequence was found 5' of the *nodYABC* operon, whereas a divergent, putative *nod* box sequence was found 5' of the *nodD1* operon. We previously proposed that this sequence was involved in *nodD1* induction. The transcriptional start sites for *nodD1* and *nodY* were determined by primer extension and found to be 44 bp and 28 bp, respectively, downstream to their respective *nod* box sequences. A series of deletions of the *nodD1* promoter were constructed and fused to *lacZ*. Analysis of the inducible activity of these deletions clearly indicated that the divergent *nod* box sequence was essential for induction by isoflavones and soybean seed extract. Based on this information, we have reanalyzed the known *nod* box sequences and found a repetitive sequence structure previously overlooked:

$$-AT^{GC}_{CG}A^{AA}_{TT}Y^{A}_{T}-AT^{GC}_{CG}A^{AA}_{TT}Y^{A}_{T}-N4-AT^{GC}_{CG}A^{AA}_{TT}Y^{A}_{T}-AT^{GC}_{CG}A^{AA}_{TT}Y^{A}_{T}-$$

E-37

Genetic analysis of two symbiotic mutants of *Bradyrhizobium japonicum*. Jong-Yoon Chun* and Gary Stacey. Dept. of Microbiology, Center for Legume Research, Graduate Program of Plant Physiology and Genetics, University of Tennessee, Knoxville, TN 37996

Two Tn*5*-induced Nod$^+$, fix$^-$ mutants of *B. japonicum* strain 61A101C were isolated. These mutants, strain ML126 and ML150, have similar symbiotic phenotypes in that they are defective in release from the vesicles. Although the original Tn*5* mutants were on distinct EcoRI fragments, subsequent cloning of these mutations are closely linked. The corresponding wild-type regions have been cloned from both strain 61A101C and USDA110. In USDA110, the regions corresponding to the original ML126 and ML150 mutations do not appear to be as closely linked as in 61A101c. Strain 61A101C and USDA110 are representatives of the *B. japonicum* Group II and Group I taxonomic subgroups, respectively. Comparative analysis of the cloned regions between these strains should be interesting with regard to the genomic organization of these two groups of *B. japonicum*. Insertion and deletion mutants have now been constructed in the cloned regions in order to better define the extent of the symbiotic region as well as the functions of encoded genes.

E-38

Characterization of a new host-inducible genetic locus in *Bradyrhizobium japonicum*. Sohail Luka* and Gary Stacey. Department of Microbiology, Center for Legume Research, University of Tennessee, Knoxville, TN 37996.

Nodulation genes in *B. japonicum* are induced by the presence of isoflavones produced by the soybean host. A Tn*3-lacZ* insertion in a locus linked to the *nodYABC* operon of *B. japonicum* was shown to be induced 8-fold by the isoflavone genistein or soybean seed extract. Sequencing of the DNA region identified by the Tn*3* insertion revealed the presence of a putative open-reading-frame (ORF) with a possible size of 1.2 kb. Additional mutations have now been constructed in this ORF and their symbiotic phenotype is currently being tested.

E-39

Construction of broad host range NPTII reporter vectors and their use in analysis of constitutive and inducible promoters in *Bradyrhizobium japonicum* USDA 110. W. Mark Barbour, Maria G. Schell, and Gary Stacey. Dept. of Microbiology and Center for Legume Research, University of Tennessee, Knoxville, TN 37996-0845, USA.

A series of broad host range vectors for making translational fusions with the protein neomycin phosphotransferase were constructed. The vectors allow direct selection of active fusions which will confer kanamycin resistance on their host. Partially digested *Sau*3A I restriction fragments from *B. japonicum* USDA 110 were ligated into the three vectors and the mixture was electroporated into USDA 110 selecting for tetracycline resistance. Clones were screened for constitutive resistance to kanamycin and active fusions were transferred to *Escherichia coli* and screened for kanamycin resistance. Both resistant and sensitive clones have been isolated, indicating the presence of promoters which function in both backgrounds. Other clones were screened for inducible kanamycin resistance in the presence of soybean seed extract and lectin. Presumptive inducible clones were then screened by western analysis using anti-NPTII antibody.

E-40

Efficient high voltage electroporation of *Bradyrhizobium japonicum*. Dennis R. Hattermann and Gary Stacey. Dept. of Microbiology and Center for Legume Research, University of Tennessee, Knoxville, TN 37996-0845, USA.

Bradyrhizobium japonicum cells were transformed with a 30 Kb plasmid to efficiencies as high as 10^7 transformants/ug DNA by high voltage electroporation. Electrical conditions resulting in the highest efficiencies were voltages of 10.5-12.5 Kv/cm and pulse lengths of 6-7 milliseconds. The number of transformants increased linearly with increasing DNA concentration over four orders of magnitude and appeared to begin saturation between 120 ng - 1.2 ug/ml. The same plasmid DNA (extracted from strain USDA 110) transformed *Bradyrhizobium* sp. (*Arachis*) with high efficiency while the same plasmid extracted from *E. coli* transformed *B. japonicum* (110) at very low efficiency. USDA 110 was also efficiently transformed with this plasmid extracted from USDA 135. Extracting plasmid DNA from a DAM-, DCM- *E. coli* strain has been shown to allow higher transformation efficiencies with selected plasmids in *B. japonicum*. We are currently testing the applicability of the methylase minus strain with several different plasmids as well as searching for a novel means of efficiently electroporating *E. coli* derived plasmid DNA into *Bradyrhizobium*.

E-41

A *Bradyrhizobium* mutant altered in host specific bacteroid development: use of the GUS gene fusion marker in genetic manipulation of bacteria.

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Bradyrhizobium strain NC92 #727 is a host specific fixation mutant of wild-type strain NC92. On one host plant, Groundnut, nodules are formed by strain #727, but symbiotic nitrogen fixation is negligible: on two other host plants, Pigeonpea and Siratro, the symbiosis is unaffected. Groundnut is unusual in having spherical bacteroids whereas those formed with Siratro or Pigeonpea are cylindrical. Ultrastructural examination of the #727-induced groundnut nodules reveals that a few spherical bacteroids do form, but they are twice the diameter of normal Groundnut bacteroids. These #727 bacteroids can fix nitrogen, but such nodules appear to senesce rapidly and the overall contribution of fixed nitrogen to the plant is very low.

Mutant #727 was isolated during transposon Tn*5* mutagenesis and contains a single Tn*5* insertion. Numerous attempts were made to introduce the Tn*5* insertion from #727 into the wild-type NC92 genome to demonstrate that the locus disrupted by Tn*5* is responsible for the observed #727 phenotype. However, the standard methods for marker exchange, the incompatible plasmid method or the use of "suicide" plasmids, both proved problematic. I have therefore begun to develop modifications of these methods that use the ß-glucuronidase (GUS) gene fusion marker to follow the movement of plasmids in *Bradyrhizobium*. This has the advantage that the presence or absence of a plasmid can be rapidly detected by a simple colour assay.

E-42

A Single *Bradyrhizobium* Locus Differentially Affects Host Plant Nodule Development

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Wye College, University of London, Wye, Ashford, Kent UK

Bradyrhizobium **strain NC92 forms an effective N_2-fixing symbiosis with three hosts, Siratro, Groundnut and Pigeonpea. A transposon mutant of NC92, #284, forms a partially effective symbiosis with Siratro and Groundnut but shows only minimal fixation on Pigeonpea. A 3.2 kb DNA restriction fragment from the wild type strain, corresponding to the region of transposon insertion, complements the mutation in #284 on Siratro and Pigeonpea, and probably also on Groundnut although secondary effects of the vector complicate results on this host. Thus, a single locus appears to be involved in this host specific phenotype.**

Energy costs and relative efficiency of symbiotic fixation are unaffected in the mutant, as is free-living fixation. However, nodulation and the onset of nitrogen fixation are delayed in all cases. Also, mature nodules have fewer infected cells and these cells show aberrant structure with fewer bacteroids and many lytic vacuoles on Pigeonpea and, to a lesser extent, on Groundnut.

These results suggest that the defect in #284 does not lie in the central process of nitrogen fixation itself, but in some aspect of host/bacterial interaction - such as signaling or metabolic compatibility - which may be of particular importance in Pigeonpea.

E-43 Random lac transcription fusion mutagenesis in Bradyrhizobium japonicum. Peter van Berkum* and Michael J. Sadowsky. USDA-ARS, Beltsville, MD 20705 and Dept. of Microbiology and Soil Science, University of Minnesota, St. Paul, MN 55108.

Besides nodulation and nitrogen fixation genes, there exists another class of genes important for the symbiosis for which no readily identifyable genotypes exist. Examples include genes involved with competition, chemotaxis, saprophytic competence, efficiency for nodulation and nitrogen fixation, and stress. The identification of these genes is essential for understanding the complex nature of the symbiosis and for the construction of improved strains. Random lac transcription fusion mutagenesis is potentially a useful approach for identifying and understanding the regulation of these genes. Existing methods are ineffective for isolating random transcription fusion mutants in B. japonicum. We developed a method for B. japonicum using the mini-mu phage MudI 1681 carrying kanamycin resistance and the lacZYA genes to generate random transcription fusions. Success depended upon which recipient strain was used. A 30% incidence of vector survival was a potential problem requiring the use of specific recipient strains and determinations for loss of vector in mutant libraries. The isolation of respiratory mutants demonstrated the usefulness of mini-mu as a transposon for mutagenesis of B. japonicum. One of four mutants responded to oxygen indicating the potential for isolating mutants by differences in expression of beta-galactosidase under treatment versus control conditions. Transconjugants with insertions in genes responding to changes in osmotic pressure were isolated using enzyme assays in microtiterplates.

E-44 *Bradyrhizobium japonicum* genes involved in the establishment of functionally intact peribacteroid membranes in soybean root nodules. P. Müller*, A. Schultes, and D. Werner. Philipps University Marburg, Dept. of Biology, 3550 Marburg, FRG.

Three genetically diverse strains of *Bradyrhizobium japonicum* previously have been shown to be symbiotically defective. The soybean nodules, induced by these strains all have in common an instability of the peribacteroid membrane. As a consequence, the infected plant tissue undergoes a complete lysis. A gene bank of *B. japonicum* 110*spc*4 based on the mobilizable broad host-range vector pVK100 was constructed in order to genetically complement the mutants to an effective phenotype. The various cosmids that were isolated by this procedure will be introduced and first experiments to characterize these specific *B. japonicum* DNA fragments are shown.

E-45

Iron regulation of the *hemA* genes from *Bradyrhizobium japonicum* and *Rhizobium meliloti*. Mary Lou Guerinot*, Erin Connolly and Barbara Anne Morisseau. Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755.

The control of rhizobial heme production is of interest as the bacterial partner is believed to synthesize the heme moiety of leghemoglobin, with the plant partner contributing the apo-leghemoglobin, thus forming a truly symbiotic protein. Iron has been implicated as a positive regulator of Δ-aminolevulinic acid synthase, the enzyme which catalyzes the first committed and rate-limiting step in the heme biosynthetic pathway. Having previously cloned and sequenced the gene (*hemA*) encoding this enzyme from *B. japonicum*, we set out to determine how the iron control was effected. Using either a *hemA-lacZ* fusion integrated into the *B. japonicum* chromosome (which leaves the resident *hemA* gene intact) or a *hemA-lacZ* fusion carried on a broad host range plasmid, there was approximately three-fold less ß-galactosidase activity in iron-deficient cells than in iron-sufficient cells of *B. japonicum*. A *glnA-lacZ* fusion served as a control; the *B. japonicum glnA* gene had previously been shown to be constitutively expressed under a variety of growth conditions. Because the *B. japonicum hemA-lacZ* fusion also showed iron regulation in *E. coli*, we have conducted experiments to determine whether this regulation is mediated by Fur, a DNA-binding protein which has been shown to regulate a number of genes in response to iron, including iron uptake genes. Indeed, iron regulation of the *hemA-lacZ* fusion is lost in an *E. coli fur* mutant. There are sequences upstream of both the *B. japonicum* and *R. meliloti hemA* genes with homology to an "iron box" as described for *E. coli*, which is the site at which the Fur regulatory protein binds to DNA. We are currently assaying plasmids carrying deletions of the "iron box" for ß-galactosidase activity in iron-deficient and iron-sufficient cells. We are also using the *B. japonicum hemA-lacZ* fusion to examine *hemA* expression *in planta*.

E-46

Nitrogen regulation in *Bradyrhizobium (Parasponia)* sp. ANU289

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Although nitrogen regulation (Ntr) of gene expression does occur in rhizobia the extent to which it occurs remains obscure.

We have recently isolated a pleiotropic, Tn5-induced mutant of *Bradyrhizobium (Parasponia)* sp. ANU289 which appears to be affected in nitrogen regulation. In contrast to the wild-type, the mutant was unable to grow on 10 mM KNO_3 as the sole source of nitrogen, nor was it able to induce nitrate reductase (NR) activity when transferred from glutamate to KNO_3. The mutant and wild-type showed similar growth kinetics when grown on either 10 mM or 1 mM NH_4Cl. However, under N-limited conditions (e.g. 10 mM glutamate) the mutant grew more slowly and in contrast to the wild-type did not display ammonium transport (Amt) activity. Furthermore, when grown under the same N-limited conditions, the mutant did not show any glutamine synthetase II (GSII) activity, although GSI activity was present at wild-type levels. The wild-type expressed both GSI (which is constitutive) and GSII (which is inducible) under these conditions. The mutant retained the ability to fix nitrogen in siratro root nodules, although development of the symbiosis was somewhat retarded.

The results suggest that at least 3 genes: those encoding GSII, Amt and NR are under the control of an Ntr system in *B. (Parasponia)*. The phenotype of the mutant is consistent with the absence of an active inducer protein such as the product of *ntr C*.

E-47

Regulation of *nifA* expression in *Azorhizobium caulinodans* ORS571. J. Stigter*, K. Pawlowski, P. Ratet, J. Schell, and F. J. de Bruijn. Max-Planck-Institut, 5000 Koln 30, F.R.G.

The *A. caulinodans* ORS571 nitrogen-fixation regulatory gene *NifA*, is controlled by the cellular nitrogen and oxygen status. Of interest is which biochemical components are involved in the transduction of the signals leading to expression of *NifA*. At least three gene products are involved in the activation of *NifA*, these are NtrC, and NtrYX. In addition FNR, supercoiling of the DNA and NifA itself have been found to influence the expression of *NifA*. The *NifA* 5' upstream region contains several DNA sequence motifs that are highly homologous to DNA elements in oxygen and nitrogen regulated promoters. These are the FNR-binding motif, NifA-binding motif (UAS) and a sequence homologous to a promoter element found in the -24 to -12 region of NtrA-dependent promoters. To analyze the involvement of the different gene products in activating the *NifA* promoter, the exact role of these *cis*-acting elements was examined. With site-directed mutagenesis (using PCR), the conserved bases of the different boxes were changed and their effect as *nifA* expression, measured with a translational *nifA-lacZ* fusion, integrated in a non-essential region of the chromosome. A model for *nifA* gene regulation in *A. caulinodans* ORS571 based on the results will be presented.

E-48

Identification and characterization of the Azorhizobium caulinodans nodD gene and of a natural nod gene inducer. D. Geelen, K. Goethals, G. Van den Eede, E. Messens, M. Van Montagu and M. Holsters. Lab. of Genetics, State University Gent, 9000 Gent, Belgium.

The stem-nodulating Sesbania rostrata symbiont ORS571 has been shown to harbour nodABC related genes the expression of which is induced in the presence of host plant exudate or commercially purchased naringenin (Goethals et al., 1989). No evidence for the presence of a nodD gene was found in the nucleotide sequence upstream of the nodABC operon.

We were able to clone a functional ORS571 nodD gene from a pLAFR1 gene library by screening for naringenin induced expression of a nodA-lacZ fusion in an Agrobacterium background. Tn5 mutagenesis of the cloned insert DNA followed by homogenotization finally resulted in a mutant strain, ORS571-3, in which the common nod genes could not be induced and that had lost the capacity to nodulate the roots and stems of Sesbania rostrata. Sequencing of the nodD locus indicated the presence of a 942-bp-long open reading frame (ORFD), with low but significant homology to the nodD genes of (brady)rhizobia. The sequence of the ORS571 nodD gene will be shown and discussed.

In addition data concerning the identification of the major naturally occurring inducer from Sesbania rostrata seedlings will be presented.

References

Goethals K., Gao M., Tomekpe K., Van Montagu M., Holsters M. (1989). Mol Gen Genet 219:289-298.

E-49

Highly divergent nod-box sequences in Azorhizobium caulinodans strain ORS571; towards a new consensus sequence. K. Goethals*, M. Gao, D. Geelen, G. Van den Eede, M. Van Montagu and M. Holsters. Lab. of Genetics, State University Gent, 9000 Gent, Belgium.

We have isolated and characterised different flavonoid inducible promoters from the stemnodulating Sesbania rostrata symbiont A. caulinodans ORS571. Sequence comparison with (brady)rhizobial nod-boxes showed only some 50% of homology. Especially the 3' end of the sequences which are very well conserved among (brady)rhizobia shows a lot of divergence in ORS571. One of the ORS571 promoters, found upstream of the common nod locus (Goethals et al., 1989) was recognised as a nod-box by the NodD protein of the wide host range strain MPIK3030. Conversly the ORS571 NodD protein (see accompanying poster by Geelen et al.) is able to recognise and activate the nod-box found upstream of the common nod operon of R. leguminosarum. We will propose a new, more stringent nod-box consensus sequence revealing some interesting repetitive motives. The implications for a NodD/nod-box interaction model will be discussed.

E-50

Isolation of the malate dehydrogenase gene from *Bradyrhizobium japonicum* 3I1B-2143 and -110. James K. Waters and David W. Emerich*, Dept. of Biochemistry, University of Missouri, Columbia, MO 65211

The carboxyl-half of the malate dehydrogenase gene from *Bradyrhizobium japonicum* 3I1B-2143 was isolated from a Lambda gt11:*Bradyrhizobium japonicum* DNA expression library. Plaques which could express the β-galactosidase/malate dehydrogenase fusion protein were detected using polyclonal antibodies specific to *B. japonicum* malate dehydrogenase. Two plaques (Lambda gt11-10 and Lambda gt11-89) were isolated which reacted to the antibodies. Lambda gt11-10 was shown to contain 456 bases and codes for amino acids of the C-terminal portion of the *mdh* gene. The DNA sequence of Lambda gt11-89 starts at the same 5' position as Lambda gt11-10. The DNA sequence codes for the entire C-terminal portion of the *mdh* gene and is extended > 400 bases downstream of the *mdh* stop codon.

The Lambda gt11-89 insert was used as a probe to screen the *B. japonicum* 3I1B-110 genomic library provided by Dr. B. Chelm. Screening of 30,000 bacterial colonies on a single plate resulted in the detection of 18 positive colonies (screening of approximately 2000 colonies results in a 99% probability that the whole genomic library was examined). Ten of these colonies were chosen at random and purified by single colony isolation. These colonies will be used to obtain the complete DNA sequence for the malate dehydrogenase gene from *Bradyrhizobium japonicum* 3I1B-110.

E-51

NADP-malic enzyme in Bradyrhizobium japonicum

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NAD- and NADP-malic enzymes play a key role to supply acetyl-CoA from C_4-dicarboxylic acid, like succinate or malate, for continous operation of citric acid cycle in nodule bacteroids[1,2].

Since the malic enzymes are not abundant proteins in soybean nodule bacteroids, Bradyrhizobium japonicum A1017 was cultured in peptone-yeast extract medium, and the NADP-malic enzyme was purified by ammonium sulfate precipitation, DEAE-cellulose chromatography, preparative IEF, hydroxyapatite chromatography, 2,5-ADP-sepharose and NAD-agarose affinity chromatography. The malic enzyme activity was eluated as one peak, and was purified to be homogeneity. The purified enzyme can decarboxylate OAA. Addition of Mn^{2+} to the reaction system was necessary after the enzyme was treated with EDTA, and 50% of recovery of the activity was observed by Mg^{2+}. NH_4^+ had no effect. The Km against to malate was 185 microM, and to NADP 4.94 microM. The nodule bacteroids contained at least two NADP-malic enzymes suggesting the symbiotic induction of a malic enzyme isozyme.

1)Kimura,I. and Tajima,S.(1989) Soil Sci.Pl.Nutr.,35:271-279
2)Tajima,S. et al.(1990) Agric.Biol.Chem.,54: in press

E-52

NAD- and NADP-dependent malic enzymes in Bradyrhizobium japonicum USDA110.

R.G. Quinnell*.L.Copeland and D.A.Day. Botany Department, Australian National University, Canberra, 2601, Australia.

It would appear that the primary substrates supplied to the bacteroids for fuelling nitrogen fixation in soybean nodules are C_4-dicarboxylates. C_4-dicarboxylate catabolism via the co-ordinated activities of both malic enzyme and the TCA cycle enzyme malate dehydrogenase provides a possible means of fulfilling the energy and carbon requirements of bacteroid metabolism. Two malic enzymes have been identified in the crude enzyme extracts of Bradyrhizobium japonicum USDA110 bacteroids, an NAD- and an NADP-dependent form; clearly, there must be a different physiological role for each. The determination of these separate roles was attempted here by an induction study on the free-living form of B. japonicum and the examination of possible in vivo effectors on the activities of each enzyme in vitro using a partially purified enzyme extract. Each enzyme 1) was found to be differentially induced according to the carbon substrate supplied; NAD-ME but not NADP-ME was induced by growth on C_4-dicarboxylates, and 2) exhibited different responses to effectors.

Activities of both NAD-ME and NADP-ME were found in the crude enzyme extracts of bacteroids isolated from Parasponia and Siratro nodules suggesting that malic enzyme activities may be an essential feature of effective Bradyrhizobium symbioses.

E-53

Bradyrhizobium japonicum δ-aminolevulinic acid (ALA) synthase is not essential for the expression of bacteroid heme in soybean root nodules. I. Sangwan and M.R. O'Brian*. Dept. of Biochemistry, SUNY at Buffalo, NY 14214, USA.

The *Bradyrhizobium japonicum* ALA synthase mutant MLG1 grew well in yeast extract-glycerol medium in the absence of exogenous ALA. No heme was discerned spectrophotometrically in extracts of those cells, and thus heme formation is strictly dependent on ALA synthase activity. Conversely, MLG1 bacteroids isolated from soybean root nodules contained significant levels of protoheme and mesoheme relative to that found in bacteroids of the parent strain I110, hence bacterial ALA synthase activity is not essential for heme expression in bacteroids. Activity of the heme synthesis enzyme porphobilinogen (PBG) deaminase was measured because the heme precursor PBG is a cofactor as well as a substrate for that enzyme in prokaryotes and in eukaryotes. MLG1 cultured cells contained significantly less PBG deaminase activity compared with that of I110, which is expected of a strain deficient in the ability to synthesize PBG *in situ*. However, MLG1 bacteroids possessed wild type levels of PBG deaminase activity, suggesting that PBG is available to the mutant bacteroids. Two possible explanations are consistent with the present findings: i) An alternate mode of ALA synthesis occurs in bacteroids that is absent in cultured cells or ii) the soybean host can provide bacteroids with ALA or PBG for heme synthesis; provision of any other heme precursor would not explain the normal PBG deaminase activity in MLG1 bacteroids. Either explanation implies that heme synthesis occurs in MLG1 bacteroids despite the absence of ALA synthase.

E-54

A strain of Bradyrhizobium japonicum with improved symbiotic properties. William J. Hunter. USDA-ARS, Soil-Plant-Nutrient Research Unit, P.O. Box E., Fort Collins, CO 80522, USA.

Bradyrhizobia with altered tryptophan metabolism often have altered symbiotic properties. However, in most cases mutants with altered tryptophan metabolism exhibit poor symbiotic properties. For this study several strains of Bradyrhizobium japonicum that exhibit altered tryptophan metabolism were studied. In culture many of these strains accumulated anthranilic acid and tryptophan degradation products, an indication of altered tryptophan metabolism. Some of the strains appeared to have enhanced tryptophan catabolism and others did not. Those strains that exhibited enhanced tryptophan catabolism accumulated the most anthranilic acid. Greenhouse studies showed that at least one strain exhibited improved symbiotic properties. Plants inoculated with this strain formed more nodules and contained more nitrogen than did plants inoculated with wild-type bacteria.

E-55

Membrane vesicles-bound nitrate reductase activity from Bradyrhizobium japonicum. M.J. Delgado, J. Olivares and E.J. Bedmar*. Departamento de Microbiología. Estación Experimental del Zaidín. C.S.I.C. 18080-Granada. Spain

A membrane-bound nitrate reductase was isolated from cells of Bradyrhizobium japonicum strain L-236 grown in defined medium with glutamate as the sole nitrogen source. This nitrate reductase was transient, with all activity being lost by late log phase. After breakage of L-236 cells by sonication, a procedure involving solubilization of the membrane proteins with Triton X-100 and filtration on Sephacryl S-200 resulted in a nitrate reductase activity capable of forming 58 nmol NO_2^- . min^{-1} . (mg protein)$^{-1}$. The relative molecular mass of the enzyme, estimated by gel filtration, was of about 200 kDa. Glutamate-grown cells also expressed nitrate reductase activity in the cytosolic fraction. Following selective precipitation with ammonium sulfate and gel filtration on Sephacryl S-200, the relative molecular mass of the soluble enzyme was of about 170kDa. Differences in molecular masses, Kms and inhibitors sensitivity between the soluble and membrane vesicles-bound nitrate reductases indicate that the two enzymes are different. Membrane vesicles-bound nitrate reductase from cells of B. japonicum L-236 grown aerobically or microaerobically with nitrate as the only nitrogen source also had a relative molecular mass of 200 kDa.

E-56

Utilization of 14-C-substrates by Bradyrhizobium lupini. V.I.Romanov*, V.I.Shramko, N.G.Fedulova, B.F.Ivanov and W.L.Kretovich. A.N.Bach Institute of Biochemistry, Leninsky pr.33, Moscow, 117071, USSR

Washed cells of the chemostat Bradyrhizobium lupini culture grown on a nitrogen-limited organic medium containing glucose (1.0%) and molasses (0.3%) utilized 14-C-sucrose 100 times more actively than that grown on a carbon-limited medium. Bacteroids isolated from Lupinus luteus L. nodules are able to utilize 14-C-organic and amino acids, as well as various sugars. The highest utilization rate of all the compounds was observed under aerobic conditions. At lower O_2 concentration (0.1-5.0%) the total incorporation of succinate, glucose and sucrose carbon into the cell and 14-CO_2 is comparable. The radiorespirometric pattern of the utilization of specifically labeled 14-C-glucose by the bacteroids depended on the O_2 concentration. At 5% O_2, $C_1 = C_6 > C_2 \geq C_{3,4}$, which suggests the existence in the bacteroids of more than one pathways of glucose catabolism. At the same O_2 concentration, the radiorespirometric pattern for succinate is typical for the Krebs cycle ($C_{1,4} \gg C_{2,3}$). The enzymes of the EMP and PP pathways were found in the bacteroids, while the enzymes of the ED pathway were almost inactive. In the Fix$^-$ bacteroids, the ED pathway was dominant.

E-57

The molecular biology of glutamine synthetase of Bradyrhizobium sp. (Lupinus). A.V. Pushkin*, E.F. Kalderon, A.G. Ivanoushkin, N.A. Solovieva, Z.G. Evstigneeva. A.N. Bach Institute of Biochemistry, 33 Leninsky pr., II707I Moscow, USSR.

It has been found 3 forms of glutamine synthetase (EC 6.3.I.2; GS) in bacteroids of yellow lupin (Lupinus luteus L.) inoculated with Bradyrhizobium sp. (Lupinus) 359a. GSIII has Mr about 600,000, consists of I2 subunits, possesses heat stability and is regulated by adenylylation. GSII has M_r about 340,000, consists of 8 subunits, possesses heat lability and is not regulated by adenylylation. Properties of GSI is not determined because of its extremely low quantity and activity. We have obtained the expression of the 3 GS forms in free living cells of Bradyrhizobium sp. (Lupinus) 359a. Now we have constructed Bradyrhizobium sp. (Lupinus) 359a genomic library and are screening it for GS genes.

E-58

A Rhizobium phaseoli Tn-5 mutant affects pleiotropically succinate utilization, GSII activity and N_2 fixation. A. Bravo, L.M. Martínez, Y. Mora, J. Calderón*, and J. Mora. Dept. of Molecular Ecology, Centro de Investigación sobre Fijación de Nitrógeno, University of Mexico, Cuernavaca, Morelos, México.

A single Tn-5 insertion mutant (AB2) of R. phaseoli CFN42 that does not grow well in NO_3^- or NH_4^+ and that is estimulated by glutamine, has been obtained. This mutant transports 40% less succinate than the parental type, expresses a very low GSII activity and has a delayed and low N_2 fixation phenotype. This new mutant utilizes glutamine preferentially than succinate as carbon source. A plasmid with the gene ntrA does not complement this mutant, neither ntrC hybridizes with the AB2 mutation. Also a plasmid with the dctABD genes does not complement this new mutation. In contrast with the parental type, the AB2 mutant although grows at a reduced rate in succinate-NH_4^+, maintains the same rate in serial subcultures in this medium (see Encarnación, S. et al.). There is a coordination between the transport of succinate and the expression of GSII since the AB2 mutant grows optimally in glucose where it expresses a high GSII activity. It is proposed that this new mutation regulates succinate transport which induces GSII and allows a burst of growth in succinate, that impairs further growth, something that allows Rhizobium to differentiate as a bacteroid and fix N_2.

E-59

Purification and Characterization of a Novel Glutamine Synthetase From Rhizobium meliloti. Yuan Liu,*[1,2] Bob Shatters,[1,3] and Michael Kahn.[1,4] Institute of Biological Chemistry[1] and Departments of Biochemistry,[2] Genetics and Cell Biology[3] and Microbiology,[4] Washington State University Pullman, WA 99164-6340

A *Rhizobium meliloti glnA glnII* mutant can grow on minimal medium that contains glutamate and ammonium, implying present of a third glutamine synthetase. We have purified an enzyme from this mutant that we call GSIII. SDS-polyacrylamide gel electrophoresis analysis of purified GSIII gives two bands of 46.5 Kd and 48 Kd. V8 protease digestion of these two bands shows that they are related proteins. The purified protein has a native MW of 355 Kd, suggesting the native enzyme is an octamer. GSIII has considerable glutamine synthetase biosynthetic activity with a pH optimum of 6.8 and a temperature optimum of 50°C but it has no detectable transferase activity. The enzyme is inhibited by ADP and pyrophosphate but not by any of the nitrogen containing metabolites that cause end-product inhibition of other GS enzymes. GSIII activity is also inhibited by methionine sulfoximine, but at concentrations much higher than are generally needed to inhibit GS enzymes. The K_m of GSIII for glutamine is 13.3 mM, for NH_4^+ is 40 mM, and for hydroxylamine is 5.3 mM. Antibodies to GSIII react with a protein of 48 Kd in *E. coli* cells that carry pFB6162, a plasmid that carries the *R. meliloti glnT* gene but we have not yet been able to detect GS activity in this strain. The role of the GSIII will be discussed in relation to both free-living and symbiotic growth.

E-60

Glutamine cycling in Rhizobia. S. Encarnación, V. Narváez, E. Martínez, Y. Mora, H. Taboada, A. Bravo, J. Calderón, and J. Mora*. Dept. of Molecular Ecology, Centro de Investigación sobre Fijación de Nitrógeno, University of Mexico, Cuernavaca, Morelos, Mexico.

It has been found that R. phaseoli biotype 1 and 2, and R. meliloti lose the capacity to grow on M.M. (succinate-NH_4^+) after being subcultivated on this medium. The addition of biotin or glutamine allows these Rhizobia to grow continually on M.M. Since R. phaseoli is able to synthesize its own biotin the growth on succinate must impose a high demand of acetyl-CoA for glutamine synthesis. The loss of growth on M.M. is different for each Rhizobium and is related to GSII activity, that also differs in isoelectric point. R. meliloti lacking GSI and GSII (De Bruijn et al., JB, 171, 1673, 1989) oxidizes less succinate than the wild strain. On the other hand, glutamine drives succinate oxidation only if glutamine can be synthesized. We propose that glutamine cycling operates in Rhizobia with a high energy expenditure resulting in the loss of the capacity to grow in succinate, something that is necessary for N_2 fixation during symbiosis. R. phaseoli CFN42 assimilates NH_4^+ by the GS/GOGAT pathway (Bravo and Mora, JB, 170, 980, 1988). This cyclic pathway expends more energy than when NH_4^+ is assimilated by the GDH-GS unidirectional pathway. When this strain assimilates NH_4^+ by the GDH-GS pathway it can be subcultivated continuously on M.M., however, N_2 fixation is reduced to 15%. (Bravo et al., JB, 170, 985, 1988).

E-61

Characterization of a periplasmic glycine betaine binding protein from *Rhizobium meliloti*. D. Le Rudulier*. K. Gloux. N. Riou and M.C. Poggi. Lab. Biologie Végétale et Microbiologie. URA CNRS 1114. Université de Nice, 06034 Nice Cedex, France.

In media of high osmolarity, *Rhizobium meliloti* uses glycine betaine (GB) as a powerful osmoprotectant. A high-affinity transport system has been characterized. The apparent K_m for GB was 5.6 and 9 µM with low- or high-salt-grown cells respectively. Maximal velocity was strongly stimulated (x 5) by elevated osmolarity (NaCl 0.3 M). Periplasmic fractions were isolated from the cells by cold osmotic shock. With shock fluid from high-salt-grown cells ^{14}C-GB-binding activity was demonstrated by a dialysis technique based on the retention phenomenon of binding proteins and also by the filter-binding procedure. The K_D for binding was 3 µM and optimum pH was between 7 and 8. Using a non-denaturing polyacrylamide gel electrophoresis with ^{14}C-GB added to the periplasmic fluid, we showed that the complexe is sufficiently stable to be detected by autoradiography. A radioactive band was found only with extract from high-salt-grown cells. Specificity of the binding was analyzed using ^{14}C-GB and other unlabeled betaines as competitors.

E-62

Nitrite reductase activity in plasmid-minus derivatives of *Rhizobium "hedysari"*. S. Casella* and A. Toffanin. Ist. Microbiologia Agraria, University of Pisa - C. S. Microbiol. Suolo, CNR. 56100-Pisa, ITALY

Some fast-growing strains of *Rhizobium "hedysari"* can reduce NO_2^- to N_2O after growing at low oxygen concentration. Nitrite reductase activity appeared not to be coupled to energy conservation as shown by the inability of cells to grow anaerobically on nitrite or to link proton translocation to nitrite reduction. It can be assumed as a detoxification mechanism since nitrite and nitric oxide are known to produce a serious inhibitory effect on nitrogenase and other critical functions (i.e.,leghemoglobine). Nitrogen fixation can occur also in induced free-living cells.

Plasmid-minus derivatives of *R. "hedysari"* strain IMAP 801 have been obtained by thermic shock. Lack of large plasmids resulted in loss of both symbiotic properties and ability to reduce acetylene *in vitro*. However they showed nitrite reductase activity comparable with that of the parent strain if properly induced under low O_2 concentration, indicating that the enzymes involved on nitrite reduction are chromosomally encoded.

From synthesis of cytochrome aa_3 found in aerobic cells, they seem to shift to the production of cytochrome *o* when grown under O_2-limited conditions. Cytochrome spectra of derivative strains and other *"true denitrifiers"* rhizobia (i.e. *Bradyrhizobium japonicum* strain USDA 6) have been also compared.

E-63 Deleterious effects of combined nitrogen on ex-planta nitrogenase activity, polysaccharide synthesis and lectin-binding ability of cowpea Rhizobium sp. JLn(c)

Bina Mody, Rustom Mody and Vinod Modi

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Cowpea Rhizobium sp.JLn(c), a symbiont of the legume Arachis hypogae, showed significant inhibition of ex-planta nitrogenase activity, polysaccharide synthesis and lectin binding ability when grown in Ashby's mannitol broth supplemented with various combined nitrogen (CN) salts (KNO_3 /NH_4Cl/NH_4NO_3/glutamine). A 20 mM concentration of either NO_3 or NH_4 resulted in complete inhibition of nitrogenase activity. Lectin binding ability as well as exopolysaccharide and capsular polysaccharide synthesis were reduced in the presence of CN salts, both under aerobic and microaerophilic conditions. The tricarboxylate cycle enzymes; viz, isocitrate dehydrogenase and malate dehydrogenase showed higher activities in the presence of CN salts suggesting the channelization of carbon towards CO_2 formation rather than for polysaccharide synthesis. This was further confirmed from the observed increase in the activity of glucose-6-phosphate dehydrogenase and respiration rates of JLn(c) cells in the presence of fixed nitrogen.

E-64 Control of cellular nitrogenase activity by the malate/ammonium-ratio in chemostat cultures of Rhodobacter capsulatus.

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The activity of cellular nitrogenase is determined by the C/N ratio in the growth substrates. In the phototrophic bacterium Rhodobacter capsulatus this result has been derived from studies employing an organic carbon source and an amino acid as source not only of nitrogen but also of additional carbon (1,2). In order to establish an exact relationship between the C/N ratio and nitrogenase activity R. capsulatus was grown in a carbon-limited chemostat gassed with nitrogen at defined malate/NH_4^+ ratios. Nitrogenase activity was determined on the basis of acetylene reduction as well as of total nitrogen fixation in situ. Variation of the malate supply (5-30 mM) at a constant NH_4^+ concentration (5 mM) in the feed medium revealed the onset of nitrogenase activity at 10 mM malate.
Variation of the NH_4^+-supply (2,5-10 mM) confirmed that nitrogenase activity became detectable at a molar ratio of 2 (malate/NH_4^+). This result was independent of variations of the growth rate from 0.04-0.088 h^{-1}.

1. Hillmer, P. and H. Gest (1977) J. Bacteriol. 129:724-731.
2. Moreno-Vivian, C., F.J. Caballero, J. Cardenas and F. Castillo (1989) Biochim. Biophys. Acta 977:297-300.

E-65 Ammonium assimilation in the phototrophic bacterium *Rhodobacter capsulatus*.

R. Borghese and J.D. Wall, Dept. of Biochemistry, University of Missouri, Columbia, MO 65211.

Nitrogen assimilation in prokaryotes has been shown to proceed via the action of glutamate dehydrogenase (GDH) or through the coupled action of glutamine synthetase (GS) and glutamate synthase (GOGAT) with the latter operating to assimilate ammonium generated during nitrogen fixation. Evidence is beginning to accumulate that alternatives to those pathways may exist. We have isolated mutants of the phototroph *Rhodobacter capsulatus* strain B100 that are lacking a measurable GOGAT activity. Because this wild-type strain does not produce significant GDH, we would anticipate that the GOGAT mutants would be strict glutamate auxotrophs. However, the mutants grow on N_2 without added glutamate. Analysis of the mutants also suggests that the GS/GOGAT assimilatory pathway functions during aerobic growth while an unidentified pathway may predominate under photosynthetic conditions. Enzymatic and physiological characterization of the mutants will be presented.

E-66 Selection of Nif$^-$ mutants in chemostat cultures of Rhodobacter capsulatus.

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When Rhodobacter capsulatus strain B10 is grown photosynthetically in N-limited continuous culture, spontaneous Nif$^-$ mutants accumulate, and eventually outgrow the wild-type strain. These spontaneous Nif$^-$ mutants contain lesions in the nifR4 regulatory gene, which is homologous to the ntrA gene of enteric bacteria. Experiments in mixed chemostat cultures, using defined nifR4 and nifH mutants, have shown that selection is related to the loss of nitrogenase synthesis and activity, and is not due to an additional regulatory effect of the nifR4 mutations. In pure cultures of B10, if the dilution rate was increased from 0.057 h^{-1} to 0.210 h^{-1}, the rate of selection of Nif$^-$ mutants decreased more than six-fold, whereas the cellular content of nitrogenase and the rate of hydrogen production by the culture remained the same. Since the specific rate of ATP synthesis would be expected to increase with increasing growth rate, whereas the specific rate of ATP hydrolysis linked to hydrogen production apparently remained constant, it can be concluded that a large part of the selective pressure for Nif$^-$ mutants results, either directly or indirectly, from a limitation in ATP synthesis.

(1) Allibert, P. et al. (1984) FEMS Microbiol. Lett. 23, 221-226.
(2) Willison, J.C. and Lac, B. (1988) FEMS Microbiol. Lett. 56, 61-66.

E-67

DNA sequence and genetic analyses of two operons of Rhodobacter capsulatus nif region A containing several genes coding for ferredoxin-like proteins.

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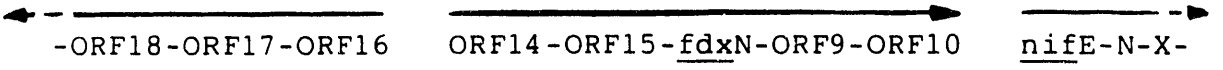
R.capsulatus nif region A contains four operons including genes homologous to nifE,N,X,Q,U,S,V,W,A,B,Z and to five nif-associated open reading frames of A.vinelandii. Sequence analysis of a 7 kb DNA fragment upstream of nifE revealed two further transcription units which are transcribed in opposite directions:



In front of each of these two operons typical nif consensus promoters were identified. However, only one NifA-binding site was found inbetween ORF16 and ORF14. Mutational analysis revealed that ORF9, ORF17 and ORF18 are absolutely required for nitrogen fixation, fdxN mutants show very slow diazotrophic growth whereas all other coding regions are not essential for the nitrogen fixation process. ORF15, fdxN and ORF17 code for low molecular weight ferredoxin-like proteins. ORF18 encodes a 56 kDa protein which contains also two cysteine motifs typical for proteins coordinating 4Fe-4S clusters. All other open reading frames identified so far in these two operons exhibit no homology to known genes.

E-68

Analysis of positive and negative regulators of *nif* gene transcription in *Rhodobacter capsulatus*.

Victor M. Pace*, Dawn Foster-Hartnett and Robert G. Kranz. Department of Biology, Washington University, St.Louis, MO. 63130

Most free-living unicellular diazotrophs activate transcription of the genes involved in nitrogen fixation only when oxygen and fixed nitrogen become limiting. The fixed nitrogen sensing regulatory circuit of the photosynthetic bacterium *R. capsulatus* is shown to be similar to the *E. coli* circuit. This involves the regulatory genes *glnB*, *nifR2* (*ntrB*-like) and *nifR1* (*ntrC*-like). The phenotype of both point and transposon insertion mutations in each gene have been used to study the regulatory circuit. *glnB* is in an operon with *glnA*, the gene encoding glutamine synthetase and mutations in *glnB* yield *R. capsulatus* strains that constitutively transcribe *nif* genes with respect to fixed nitrogen. It is shown that *nifR1* is required to activate *glnBA* (50%) transcription and another *nif* regulatory gene *nifA* (100%). We are currently defining the *nifR1* upstream activation sequence and determining potential RNA polymerase holoenzyme interactions. NIFA, another DNA-binding activator protein and NIFR4 (an NTRA-like sigma factor) subsequently activate transcription of all other *nif* genes. *nifR4* encodes a unique RNA polymerase sigma factor in that it is linked to *nifHDK* and the only effect of either *nifR4* insertion or deletion mutations is the inability to transcribe *nif* genes. A consensus sequence for the binding site of the *nifR4* sigma factor has been derived.

E-69

Organisation of nif genes in Rhodobacter capsulatus: Identification of an alternative nitrogenase system. W. Klipp*, M. Schmehl, U. Schramm, C. Moreno-Vivian+, S. Hennecke and B. Masepohl. Lehrstuhl für Genetik, Universität Bielefeld, FRG. + Departemento de Bioquimica, Universidad de Cordoba, Spain.

DNA sequence and genetic analyses of R.capsulatus nif region A revealed four transcription units containing coding regions homologous to nif genes from K.pneumoniae: nifE-N-X-ORF4-ORF5 (ferredoxin)-nifQ-ORF8, ORF6-nifU-S-V-W, nifA copyI, nifB-ORF1-nifZ. R.capsulatus NifU differs from the nifU gene products of K.pneumoniae and Azotobacter vinelandii. Only the C-terminal part (73aa) is conserved in NifU of all three organisms whereas the large N-terminal part, which is highly homologous between K.pneumoniae and A.vinelandii, is not present in R.capsulatus NifU. In addition to the "common nif genes", five open reading frames were identified which had their counterpart only in A.vinelandii. An R.capsulatus nifHDK deletion mutant is unable to fix nitrogen in the presence of molybdenum. However, slow diazotrophic growth was observed for this mutant strain in molybdenum-free media indicating the existance of at least one alternative nitrogenase system. Since this nitrogenase activity is not stimulated by vanadium it is assumed that the alternative nitrogenase of R.capsulatus corresponds to the third system identified in A.vinelandii.

E-70

PURIFICATION OF A PYRUVATE OXIDOREDUCTASE FROM NITROGEN FIXING Rhodospirillum rubrum

Erica Brostedt* and Stefan Nordlund, Department of Biochemistry, Arrhenius Laboratories, University of Stockholm, S-106 91 Stockholm, Sweden.

The components of the electron transport to nitrogenase are known in detail only for one organism, Klebsiella pneumoniae. In this bacterium pyruvate is oxidized by a pyruvate:flavodoxin oxidoreductase from which electrons are transferred to flavodoxin, the direct electron donor to dinitrogenase reductase. In K. pneumoniae these proteins are specific for nitrogen fixation, encoded by the genes nifJ and nifF respectively. The purification, molecular properties and studies on the interaction with nitrogenase of the proteins from K. pneumoniae have been reported.

In the poster we report on the purification of a pyruvate oxidoreductase from the photosynthetic bacterium Rhodospirillum rubrum grown under nitrogen fixing conditions. The procedure includes ion-exchange chromatography, dye-ligand chromatography and gelfiltration. The molecular properties of the enzyme as well as studies on the reaction catalyzed will be reported.

E-71

Glutamine synthetase from Rhodospirillum rubrum is adenylyated and ADP-ribosylated in vitro. Diana Woehle* and Paul W. Ludden. Dept. of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA.

When the supernatant fraction of R. rubrum cells is incubated with either [α-^{32}P]NAD or [α-^{32}P]ATP and the proteins separated by two-dimensional gel electrophoresis (2-D gel), ^{32}P-labelled glutamine synthetase (GS) of different mobilities is seen. ^{32}P-labelled GS purified from cells grown in low phosphate glutamate/malate medium with 1 mCi $H_3{}^{32}PO_4$ appears on a 2-D gel as three spots: one unlabelled, presumably unmodified GS, and two labelled spots, themore abundant spot migrating at the position of [α-^{32}P]ATP-labelled GS and the smaller spot at the position of [α-^{32}P]NAD-labelled GS. A ^{32}P-labelled peptide purified from a subtilisin digest of in vivo ^{32}P-labelled GS has the amino acid sequence L-(D)-Y-L-P-P-E-E-L-M, resembling the sequence around the adenylyated tyrosine of GS from Escherichia coli. The tyrosine of the R. rubrum peptide is modified; the identification of the aspartate is uncertain due to interference from ammonium. The ADP-ribosyltransferase that modifies GS does not appear to be the dinitrogenase reductase ADP-ribosyltransferase involved in nitrogenase regulation in R. rubrum. The site and effect of ADP-ribosylation on GS are being studied, and purification of the ADP-ribosyltransferase is being carried out.

E-72

Regulation of *Rhodospirillum rubrum* DRAT and DRAG proteins expressed in *Klebsiella pneumoniae ntr* and *nif* mutants. H.J. Wirt*, G.P. Roberts, and P.W. Ludden. Dept. of Biochemistry, the University of Wisconsin, Madison, WI 53706.

Nitrogenase activity in the purple nonsulfur bacterium *Rhodospirillum rubrum* is reversibly regulated by covalent modification of dinitrogenase reductase with an ADP-ribose moiety in response to NH_4^+, glutamine, and darkness in a process called "switch-off". The enzymes which catalyze the modification and demodification of dinitrogenase reductase are Dinitrogenase Reductase ADP-ribosyl Transferase (DRAT) and Dinitrogenase Reductase Activating Glycohydrolase (DRAG). The transferase and glycohydrolase have both been purified and their genes have been cloned and sequenced. The unrelated nitrogen-fixing enteric bacterium *Klebsiella pneumoniae* does not show post-transcriptional regulation of nitrogenase activity by ADP-ribosylation; however, when the DRAT and DRAG proteins from *R. rubrum* are expressed in *K. pneumoniae* on plasmid vectors , "switch-cff" regulation of *K. pneumoniae* nitrogenase is observed, suggesting that the mechanism of control of the transferase and glycohydrolase activity is highly conserved. The expression, function, and apropriate regulation of DRAT and DRAG has been tested in various *ntr* and *nif* mutants in *K. pneumoniae*.

E-73 STUDIES ON THE METABOLIC REGULATION OF NITROGENASE ACTIVITY IN Rhodospirillum rubrum

Abdelhamid Soliman* and Stefan Nordlund, Department of Biochemistry, Arrhenius Laboratories, University of Stockholm, S-106 91 Stockholm, Sweden.

Nitrogenase activity in the photosynthetic bacterium Rhodospirillum rubrum is regulated on metabolic level. This control is manifested as the inhibition of activity upon addition of effectors, e.g. ammonium ions, the "switch-off" effect. This effect is due to covalent modification of dinitrogenase reductase by, as shown by Ludden & coworkers, ADP-ribosylation catalyzed by DRAT (dinitrogenase reductase ADP-ribosyl transferase). The reactivation is catalyzed by DRAG (dinitrogenase reductase activating glycohydrolase). Although a number of compounds have been tested, the metabolic signal regulating the inter-play between DRAT and DRAG has as yet not been identified.

Addressing this question we have studied the effects of NAD on the in vivo activity of nitrogenase in Rsp. rubrum. Both the reduced and the oxidized forms of NAD(P) as well as analoges have been investigated. The effects are very similar to those obtained by Ludden & coworkers, with purified DRAT indicating that the NAD^+-pool might be part of the regulatory signal. The results will also be discussed in relation to the efflux of Ca^{2+} during "switch-off", as we have reported previously.

E-74 Construction and Characterization of Mutations in Genes Coding for the Reversible ADP-ribosylation System of Dinitrogenase Reductase from *Rhodospirillum rubrum*.

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The activity of nitrogenase in *R. rubrum* is regulated by reversible ADP-ribosylation of dinitrogenase reductase in response to exogenous nitrogen sources, or to the light energy. The genes encoding dinitrogenase reductase ADP-ribosyl transferase (*draT*) and dinitrogenase reductase activating glycohydrolase (*draG*) have been cloned and sequenced. In this work, a Kan^r cassette, containing transcriptional terminators in both ends, was cut out of pHP45 Kn with *Eco*R1 and inserted into *draG* at *Eco*RV site, and into *draT* at *Sac*II site (ends blunted when necessary). Removal of the cassette in *draG* by *Bam*H1 digestion and re-ligation leaves a 24-base pair fragment of the original cassette sequence, encoding an aa-sequence of AsnSerArgGlySer-ProGlyIle, between asp-82 and ile-83 of the DRAG sequence. Removal of the cassette in *draT* by *Sma*I digestion and re-ligation results in the replacement of the arg-191 of the DRAT sequence with GluPheProGlyIle. Both of these oligonucleotide insertions are in-frame. *R. rubrum* strains carrying mutations in *draG* and *draT* (both polar and non-polar) exhibited the same growth rate and the same pigmentation as that of wild type cells. When grown in glutamate/malate medium, the $DraT^-$ mutants had C_2H_2 reduction activity comparable to that of wild type cells. Addition of NH_4Cl to a final concentration of 2 mM, or placing the cells in the dark, did not cause the switch-off of the nitrogenase activity, while it did in the wild type cells. The dinitrogenase reductase was not modified, as indicated by the absence of ^{32}P in the protein when quickly extracted from these cells. No difference was observed between the polar and non-polar insertion mutants. Characterization of the phenotype caused by *draG* mutations is in progress.

E-75 Integration Host Factor Binds to *nif* Promoter-Regulatory Regions from a Variety of 'Purple' Bacteria. T. Hoover *, E. Santero, S. Porter, and S. Kustu. Departments of Plant Pathology and Cellular and Molecular Biology, University of California, Berkeley, CA, USA 94720.

We recently demonstrated that the integration host factor (IHF) binds to the *Klebsiella pneumoniae nifH* promoter-regulatory region [Santero et al. (1989) Proc. Natl. Acad. Sci. USA 86, 7346-7350], and that it stimulates activation of transcription by the nitrogen fixation-specific activator NIFA. We report here that IHF also binds to the *nifJ*, *nifE*, *nifU*, and *nifB* promoter-regulatory regions from *K. pneumoniae*, but not the *nifF* or *nifL* promoter-regulatory regions. In addition, IHF binds to the *nifH* promoter-regulatory regions of *Azotobacter vinelandii*, *Rhizobium meliloti*, *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Thiobacillus ferrooxidans*. In all cases where IHF binds, its binding site is located between an upstream binding site(s) for NIFA and the promoter, a binding site for σ^{54}-holoenzyme. The length of the region protected by IHF from DNase I or hydroxyl radical cleavage indicates that IHF bends the DNA; this inference is confirmed by direct electron microscopic observation. We hypothesize that the role of the IHF-induced bend is to stabilize a DNA conformation that facilitates productive contacts between NIFA and σ^{54}-holoenzyme. Based on studies at the *K. pneumoniae nifH* promoter, we anticipate that IHF will stimulate NIFA-mediated activation of transcription at many *nif* promoters.

E-76 Morphology and nitrogenase activity of derepressed *Frankia* in continuous culture. Sharon Harris* and Warwick Silvester, Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

Derepression of nitrogenase in batch cultures of *Frankia* by withdrawal of combined nitrogen, is normally followed by a peak in nitrogenase activity which is sustained for only 24 h or less. We have grown *Frankia* (ArI_3 and CcI_3) in continuous derepressed culture, with constant levels of protein and nitrogenase activity, for up to four weeks by daily addition of sterile media cultures at a nominal dilution rate (D) of 0.12 day^{-1}. Maintenance of *Frankia* in continuous culture allowed the effect of O_2 on vesicle morphology and nitrogenase activity to be studied independent of changes due to culture age and condition. When nitrogenase was eliminated by increasing the pO_2 from 3 to 21 kPa O_2, those cultures returned to low pO_2 soon began recovery of nitrogenase activity while those cultures remaining at 21 kPa O_2 showed a 36 h lag before activity returned. Adaptation to the elevated pO_2 was accompanied by development of a thickened vesicle wall and stalk and an increase in the pO_2 optimum for nitrogenase activity. Measurement of nitrogenase and respiratory activity using continuous flow techniques showed *Frankia* also responds to short term changes in pO_2.

E-77 CHARACTERIZATION OF ACTINORHIZINS INVOLVED IN THE DEVELOPMENT OF *FRANKIA-ALNUS* SYMBIOSIS.

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The objective of this investigation is to seek actinorhizal specific genes, by looking at differential expression during the development of the *Frankia*-induced nodule (actinorhiza) The polypeptide patterns of infected and uninfected *Alnus glutinosa* roots have been compared. Analysis of soluble proteins by SDS-PAGE showed an increased accumulation of three polypeptides of molecular weight 36, 50 and 61 Kd. Characterization of these polypeptides was done by Western blotting analysis using different antisera. The implication of these polypeptides in morphological and physiological changes resulting from the establishment of actinorhizal symbiosis will be discussed. Molecular cloning of actinorhizin genes by screening a cDNA library constructed from infected tissues was also undertaken. Results obtained will be presented.

E-78 **Characterization of intercellular matrix during intercellular infection of *Ceanothus* spp. by *Frankia*.** Q. Liu* and A.M. Berry. Department of Environmental Horticulture, University of California, Davis, CA, 95616.

Early infection of *Ceanothus* spp. by *Frankia* was characterized by an extensive darkly-staining intercellular matrix, prominent in the root cortex and gradually decreasing through a transition zone to the nodule cortex. *Frankia* was observed within the matrix. The common occurrence of a similar matrix in both *Frankia* and *Rhizobium* induced intercellular nodulation indicates an important function in the intercellular infection process. The nature of this matrix was examined with histochemistry, immuno-cytochemistry, and electron microscopy. Most of the matrix was composed of fibrillar electron dense material. Holes or spaces occurred in the electron dense matrix, often in conjunction with apparent changes in wall layers. Fluorescent hybridization probe analysis indicated that the matrix contains methylated and some non-methylated polygalacturonate. Preliminary results using antipolygalacturonic acid antibodies suggested that labelling occurred mainly in the middle lamella and the intercellular matrix, but not in the cell wall.

E-79

Identification of Bacteriohopanetetrol in *Frankia*. A.M. Berry*,[1] R.A. Moreau,[2] A.D. Jones,[3] and S.F. Osman.[2] [1]Department of Environmental Horticulture, University of California, Davis, CA, 95616; [2]Eastern Regional Research Center, USDA, ARS, Philadelphia, PA, 19118; [3]Facility for Advanced Instrumentation, University of California, Davis, CA, 95616.

An unusual class of lipid with amphiphilic properties has been detected in nodule tissue of *Alnus* and *Ceanothus*. Very high levels of the same lipid (20-50% of total cell lipids) were detected in solvent extracts of *Frankia* spp. cells. However, the lipid was absent in host roots. The lipid was purified and quantified by HPLC/FID (Moreau et al., in press, Phytochemistry). Phenolsulfuric acid determinations and proton n.m.r. indicated that the purified lipid is not a glycolipid. Mass spectra of the predominant species are consistent with published spectra for bacteriohopanetetrol ($C_{35}H_{78}O_4$), a pentacyclic triterpenoid, or hopanoid. Hopanoids may play a role in membrane stabilization and/or oxygen exclusion important for nitrogen fixation in *Frankia*.

E-80

Physiology and infectivity of Frankia isolates from alder and casuarina. M. Valdes* and R. Del Valle. Dpto. de Microbiología, Esc. Nacional de Ciencias Biológicas Instituto Politécnico Nacional. 11340 México, DF.

The isolation of two strains of Frankia were made from root nodules of Alnus acuminata ssp glabrata and two other strains were isolated from Casuarina equisetifolia . All strains are physiologically different; those isolated from alders are much slow growers than those from casuarinas. They also differed in their utilization of carbohydrates; growth curves for all strais in medium S show that addition of Tween to the culture medium with some sugars has the effect od depressing growth and with others has a synergistic effect. In vitro acetylene reduction is much higher among the casuarina isolates than the alder isolates. All Frankiae effectively nodulate their original hosts; questions are raised about the specificity of the various isolates.

E-81

Electroporation of Frankia spp.. B. Cournoyer* and P. Normand. Centre de Recherche en Biologie Forestière, Dept. de Foresterie, Université Laval, Québec, G1K-7P4, Canada.

Frankia spp. are slow-growing actinomycetes able to fix dinitrogen and enter in symbiosis with dicotyledeneous plants such as members of Rosaceae, Casuarinaceae and Betulaceae families. Genetic transformation of these filamentous bacteria has not been achieved, hindering studies on plant specificity, gene expression and regulation, etc... Attempts using protoplasts(1) failed due to low regeneration and lack of an efficient vector. We are trying to develop a method for studying plasmid stability and expression taking these facts in account. Electroporation(2) is becoming the favourite choice for introducing plasmids and other macromolecules in most cell types. We have introduced various macromolecules in intact Frankia cells using this approach. The parameters were defined using 70 kDa dextran molecules labeled with fluorescein-thiocyanate and confirmed with radioactively labeled DNA. Several types of plasmid DNA were introduced and their stability evaluated. Time to return under normal cellular activity after an electrical pulse was also determined.

(1) P. Normand, pers. comm.; (2) Biophys. J. 13 (1973): 1005 and Biochimie 70 (1988): 579.

E-82

Analysis of the glnII gene from the actinorhizal symbiont Frankia CpI1. D. A. Rochefort* and D. R. Benson. Dept. of Molecular and Cell Biology, Univ. of Connecticut, Storrs, CT, Storrs, CT 06269-3044, USA.

Frankia, a gram-positive actinomycete, is the N_2-fixing symbiont of a variety of non-leguminous woody plants that are involved in land reclamation and in the colonization of nitrogen-poor soils. Frankia synthesizes two glutamine synthetases (GSs), GSI and GSII. GSII has been found in bacteria that form symbioses with plants. The cloned GSII gene (glnII) from Bradyrhizobium japonicum was used to identify homologous sequences from Frankia sp. strains CpI1, CcI3, and Ai17. A putative Frankia CpI1 glnII gene was cloned into pUC19 from a size selected KpnI library. Once localized by restriction mapping, the gene was sequenced. The sequence has strong similarity to B. japonicum and Rhizobium meliloti glnII, with the high GC content expected from actinomycete DNA. Deletion of the Frankia promoter region allowed the glnII gene to be expressed from the lac promoter of pUC19 in an E. coli glnA deletion strain. These studies have important implications for the origin of the glnII gene and may provide a key to understanding the nature of N_2-fixing root nodule symbioses.

E-83

Nitrogenase genes in *Frankia* HFPCcI3. A. Reddy* and A.M. Hirsch. Dept. of Biology, U. C. L. A., Los Angeles, CA 90024, USA

We are studying symbiotic genes in *Frankia* strain HFPCcI3 which forms a nitogen-fixing association with the roots of the important actinorhizal tree *Casuarina cunninghamiana*. The nitrogenase structural genes (*nifHDK*) are conserved among a number of diverse nitrogen-fixing bacteria, including *Frankia*. We have isolated a clone, pAR2, from a partial genomic library of HFPCcI3 which contains the *nif* genes. The *nifHDK* genes are contained on a 5.6 kb *Bam*HI fragment in HFPCcI3, and the restriction map of this fragment is as expected for a *Casuarina* isolate. We have localized the different nitrogenase genes to specific restriction fragments in pAR2, and they appear to be contiguous. DNA:DNA hybridization experiments indicate that the *Frankia nifH* and *nifD* genes exhibit approx. 70% and 65% sequence similarity, respectively, to *nifH* and *nifD* probes from *Rhizobium meliloti*, while the *nifK* gene is approx. 60% homologous to a *Klebsiella pneumonia nifK* probe. We are in the process of sequencing the *nif* genes in pAR2. We intend to characterize these genes in terms of their sequence, as well as their transcriptional organization and control.

E-84

Use of rrn and nif genes from Frankia as targets for PCR detection. P. Normand[1]*, P. Simonet[2], B. Cournoyer[1] and S. Nazaret[2]. 1: Centre de Recherche en Biologie Forestière, Univ. Laval, Québec, Canada; 2: Lab. de Microbiologie des Sols, Univ. Lyon, France.

The actinomycete Frankia has established an ecologically important nitrogen-fixing symbiosis with 20 genera of woody pioneer trees and shrubs. We have sought to develop DNA probes from gene banks to study the microbe in the field. EMBL3 clones for two genomic regions, the nifHDK (1) and the rrn regions were isolated and characterized. The number of ribosomal clusters was two in strain ORS020606, and both copies were found to have the gene order 16S-23S-5S. The complete nucleotide sequence of one rrn operon was determined and by comparison with published sequences from other microorganisms, conserved sequences were chosen as target in both regions. The nifHD region was investigated first (2) and we found that oligoprobes could discriminate between PCR-amplified target DNA. However, it can be argued that nif are plasmid-borne in Frankia (3) and that one may be following transferable plasmids. We therefore decided to use rrn targets which are generally considered chromosome-borne and to sequence one hypervariable region of 280 bp in the 16S gene of 30 Frankia strains. A tentative phylogeny of the genus was thus devised and "specific" sequences are being used in field tests.

(1): FEMS Microbiol. Lett. 55: 141-146; M.G.G. 213: 238-246; J. Mol. Evol. 29: 436-447. (2): Arch. Microbiol. in press. (3): M.G.G. 204: 492-495

E-85

A linear plasmid in two Frankia strains.

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In a previous work (S.V.Dobritsa and O.S.Stupar, 1989, FEMS Microbiol.Lett. 58:287-292), a multi-copy plasmid has been detected by electrophoresis of total cellular DNA from strains S14 and S15 of a nitrogen-fixing actinomycete, Frankia sp., isolated from Shepherdia argentea root nodules. The plasmid is transmitted to the progeny during endophytic passages of the strains through host plants. In this report I show that the plasmid is represented by linear DNA molecules. The plasmid DNAs from strains S14 and S15 do not differ in their sizes (the plasmid is about 15 kb in length) and in restriction fragment patterns. The linear plasmid appears to be linked to protein since the DNA displayed increased electrophoretic mobility after treatment with proteinase K. A restriction endonuclease map of the plasmid for a few enzymes was determined. The plasmid I describe here is the first example of linear plasmids from Frankia spp.

E-86

The role of molecular chaperones in *nif* regulation and nitrogenase assembly.

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The emerging role of chaperonins, a class of molecular chaperones, in promoting correct protein folding and supramolecular assembly has led us to examine the potential function of GroE (the prototype bacterial chaperonin) in the biogenesis of the *Klebsiella pneumoniae* nitrogenase. An *E. coli groEL* mutant transformed with the *K. pneumoniae nif* cluster, and derepressed for nitrogenase formation, contained drastically reduced levels of native nitrogenase components and of their polypeptide subunits as compared with the similarly transformed, isogenic wild type strain. The two strains were also compared in pulse labeling experiments and in β-galactosidase activity expressed from several *nif'-'lacZ* fusions, or from the host *lac* operon. The results indicated the *groEL* mutation decreased *nif* expression but not *lac* expression. Further experiments showing the acceleration of *nif* derepression in *K. pneumoniae* by overexpression of the *E. coli groE* operon further support the role of the chaperonin in *nif* regulation. The possibility that chaperonins are also involved in nitrogenase assembly was examined in pulse-chase analyses of fully derpressed *K. pneumoniae* . The results provided evidence for the involvement of a high MW intermediate(s) in MoFe protein formation, a hallmark of chaperonin-dependent assembly processes.

E-87 Involvement of IHF in gene expression from the *nifH* promoter of *Klebsiella pneumoniae*.E. Santero*, T. Hoover and S. Kustu. Depts. of Plant Pathology and Molecular and Cellular Biology. University of California, Berkeley, CA 94720, USA.

The regulatory protein NIFA activates transcription of nitrogen fixation operons by σ^{54}-holoenzyme. We recently demonstrated that NIFA activates transcription from the *nifH* promoter in vitro. We also showed that integration host factor (IHF), a small protein that bends DNA in a site-specific manner, bound to this promoter region at a site located between the upstream binding site for NIFA and the promoter, a binding site for σ^{54}-holoenzyme.

We report here that IHF greatly stimulates NIFA-mediated activation of *nifH* transcription in vitro. IHF failed to stimulate transcriptional activation by the functionally related activator NTRC, which has no specific binding site in the *nifH* promoter region. Although the IHF binding site is located just upstream of the *nifH* promoter, the activator specificity suggested that IHF does not directly stimulate binding of σ^{54}-holoenzyme; this is confirmed by direct DNase I protection assays. Our evidence is consistent with the view that an IHF-induced bend facilitates contacts between NIFA and σ^{54}-holoenzyme, both of which bind to their sites independently of IHF. We will discuss experiments which indicate that the use of a "weak" *nifH* promoter plus IHF confers high fidelity of activation by NIFA together with high efficiency of activation.

E-88 **Nodulation genes in *Azospirillum*.** Vieille*, C. and Elmerich, C. Unité de Physiologie cellulaire, Dept. des Biotechnologies, Institut Pasteur, Paris 75724 Cedex 15, France.

Homology was previously detected between total DNA of several *Azospirillum* strains and restriction fragments containing *Rhizobium meliloti* nodulation genes. From *A. brasilense* Sp7, a 10 kb *Eco*RI and a 4 kb *Sal*I fragments sharing homology with a 6.8 kb *Eco*RI fragment carrying *nodGEFH* and part of *nodP* of *R. meliloti* 41 were cloned in pUC18, to yield pAB502 and pAB503 respectively.

The nucleotide sequence of a 3.6 kb *Eco*RI-*Sma*I fragment of pAB502 insert revealed 60% homology with *R. meliloti nodP* and *nodQ* genes. The two genes overlapped on one bp suggesting that they are cotranscribed. A translational *nodP-lacZ* fusion was constructed in the broad host range plasmid pGD926. No ß-galactosidase activity was detected in *E. coli* but the fusion was fonctional in *Azospirillum*, and constitutively expressed. The *nodQ* product shares homology with a family of initiation and elongation factors. More particularly, the consensus for GTP-binding site is well conserved.

The nucleotide sequence of the 1.4 kb *Pst*I-*Sma*I fragrnent of the pAB503 insert revealed 57% homology with *R. meliloti nodG*. The two *nodG* products share homology with the *Klebsiella aerogenes* ribitol dehydrogenase. Fonctional analysis of *nodG* is under progress.

In contrast with *R. meliloti* where *nodGPQ* are contiguous in *Azospirillum nodPQ* are located on a 90 MDa plasmid (P90) and *nodG* is chromosomal.

E-89

Sequence and functional analysis of the FixA gene of Azotobacter vinelandii. R. Wientjens* and H. Haaker. Dept of Biochemistry, Agricultural University, Dreijenlaan 3, Wageningen, NL-6703HA, The Netherlands

The FixA region of Azotobacter vinelandii is found on a 7 kb fragment of EcoRI digested chromosomal DNA.

From this fragment a 1.7 kb region was subcloned containing the FixA gene and the N-terminus of the FixB region. This 1.7 kb fragment was sequenced. The complete sequence will be presented.

A kanamycin cassette was inserted in the fixA gene in order to mutate the gene.

The phenotype of this mutant and the results of the physiological analysis will be discussed.

E-90

Site-directed mutagenesis of the MoFe protein (Kp1) of Klebsiella pneumoniae nitrogenase. H.M. Kent, C. Gormal, M. Buck* and B.E. Smith*. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, Sussex, U.K.

Using directed mutagenesis amino acid substitutions have been made in the α- and β-subunits of Kp1 at positions normally occupied by conserved cysteine[1] or tyrosine residues. The mutants have been analysed in terms of their growth on N_2, C_2H_2 reduction, the accessibility of FeMoco, their e.p.r. spectroscopy and by non-denaturing gel electrophoresis followed by probing with both polyclonal and monoclonal antibodies. Nif^+, Nif^- and intermediate phenotypes have been obtained and have provided evidence that Cys275 in the α-subunit and nearby amino acids are involved in binding FeMoco. Extracts of all Nif^+ mutants exhibited a normal e.p.r. signal and a distribution of electrophoretic forms comparable to the wild-type, the abundance of the major form correlating with the specific activity of the extract. Nif^- mutants exhibit no or an altered e.p.r. signal and either a major form of low electrophoretic mobility or a fast-moving form. Two single mutations, C89A in the α-subunit and C94A in the β-subunit, which are tightly Nif^- can be combined to produce a weakly active nitrogenase indicating important interactions between subunits.

[1] H.M. Kent et al. (1989) Biochemical Journal 264, 257-264.

E-91

Cytochrome d deficient mutants of Azotobacter vinelandii can fix nitrogen microaerobically but not aerobically.

M. Kelly*[2], R. Poole[2], G. Yates[1] and C. Kennedy[1]. [1]AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K. and [2]Division of Biosphere Sciences, Kings College, University of London, London, W8 7AH, U.K.

Azotobacter vinelandii DNA fragments hybridized to the Escherichia coli cydA and cydB genes, encoding the cytochrome bd terminal oxidase complex. The cydA- and cydB-like A. vinelandii regions were contiguous and apparently transcribed from cydA to cydB. cydB insertion mutations integrated into the genome resulted in A. vinelandii strains that were unable to grow on N-deficient medium when incubated in air but could grow on agar plates incubated under an atmosphere of 1.5% oxygen. Mutants showed loss of spectral features associated with cytochromes b_{595} and d. A region adjacent to cydA-like DNA was involved in the expression of cytochrome d; mutations resulted in a five-fold increase in cytochrome d levels. It is proposed that the cytochrome d branch of the respiratory chain supports the high respiration rates required for respiratory protection of nitrogenase.

E-92

Involvement of the regulatory genes nifA, vnfA, nfrX, ntrD and the structural genes nifUSVM in expression and activity of the three nitrogenases in Azotobacter vinelandii.

J. Walmsley, A. Bali, P. Woodley and C. Kennedy*. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

Expression of lacZ fusions in the structural genes for the three nitrogenases of A. vinelandii [nifH (Mo nitrogenase); vnfH, vnfD, vnfE (V nitrogenase); and anfH (third nitrogenase)] recombined into the chromosomes of regulatory mutants have led to a model describing the interacting roles of regulatory genes. nifA, nfrX and ntrD are necessary for expression of nifH. The ntrD gene was previously identified as nifA but is shown to be different by physical and phenotypic criteria. nfrX and ntrD are required for expression of the anfHDGK operon, but not for vnfH or vnfDGK expression. The product of vnfA is required for vnfH, vnfDGK and vnfEN expression in A. vinelandii. When constitutively expressed in E. coli, VnfA activated expression of vnfH and vnfEN but not vnfDGK. We previously showed that nifM product was required for the activity of all three nitrogenases and now report that products of the nifUSV genes are also necessary for growth on N_2 in the presence of Mo, or V, or of neither metal.

E-93

Mutations in a *nifL*-like gene upstream of *nifA* in *Azotobacter vinelandii* result in molybdenum nitrogenase expression in the presence of ammonia and in the absence of molybdenum. A. Bali* and C. Kennedy. AFRC Institute of Plant Science Research Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

The complete sequence of a gene upstream of *nifA* in *A. vinelandii* with some homology to the *nifL* gene of *Klebsiella pneumoniae* will be presented. Mutations in this gene resulted in the expression of molybdenum nitrogenase activity (and β-galactosidase in a *nifH-lacZ* fusion background) in the presence of normally repressive concentrations of fixed nitrogen (ammonium acetate or ammonium acetate plus urea). The *nifL*$^+$ strain is unable to synthesize nitrogenase in the absence of molybdenum; the *nifL*$^-$ strain was, however, able to express a *nifH-lacZ* fusion under this condition. Unlike in *K. pneumoniae*, expression of *nifA* occurred in the presence of ammonium or in an *ntrC* mutant (as expected from the previously reported Nif$^+$ phenotype of *ntrC* mutants). Also, *nifA* expression was at least partially independent of expression of the upstream *nifL*-like gene and did not require *rpoN*.

E-94

Identification and characterization of *nifH* promoter region in *Azospirillum lipoferum*. A. Tripathi* and W. Klingmüller. Dept. of Genetics, University of Bayreuth, 8580 Bayreuth, FRG. *Dept. of Botany, University of Gorakhpur, Gorakhpur, INDIA.

Cloning of *Azospirillum lipoferum nifHDK* region into vector pSUP106 has earlier been reported. The recombinant plasmid pFS191 complemented nitrogenase activity in *K. pneumoniae nifH*$^-$, *nifD*$^-$ and *nifK*$^-$ mutants. In order to study regulatory elements controlling *nifH* transcription, we have identified a 1.1 kb BamH1 EcoRI fragment of pFS191 which contained 5' end of the *nifH* gene and upstream region. Transcriptional fusion of this fragment with a promoterless *lacz* gene showed a strong NifA dependent activation in NtrA$^+$ background. Inability to get promoter activity with cloned *K. pneumoniae ntrC* and *nifA* genes in NtrA$^+$ and NtrA$^-$ background, respectively, indicated the requirement of NifA and NtrA for activation of *A. lipoferum nifH* promoter. Nucleotide sequencing of the cloned fragment (in progress) may reveal the presence of putative NtrA dependent promoter and NifA dependent upstream activating sequences.

E-95

Tn5-induced mutants of Azospirillum brasilense ATCC 29710 (Cd) altered in cell surface properties.

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Azospirillum brasilense ATCC 29710 (Cd) was mutagenized with Tn5 via a suicide vector plasmid pRK602 at a frequency of 2.1×10^{-7}. Mutants were selected on nutrient agar containing kanamycin and streptomycin. Analysis of 700 kanamycin resistant mutants yielded two Tn5 derivatives impaired in nitrogenase activity (C_2H_2 reduction) in N-free semisolid malate medium. Mutant Cd.6A expressed reduced rates of growth and pellicle formation while mutant Cd.16A did not grow at all nor formed pellicle even after prolongued incubation (5 days). However, both mutants exhibited similar growth patterns as the wild type in liquid and solid rich media under aerobic conditions. Cd.16A was defective in exocellular polysaccharide (EPS) synthesis as indicated by the fai lure to yield fluorescence in calcofluor containing agar plates (Cal minus). Flocculation in minimal medium containing fructose and nitrate(MMFN) was significantly reduced by about 70% and nitrite accumulated in the medium (140 nmol NO_2^- / mL). Mutant Cd.6A compared well with the wild type in terms of EPS production, calcofluor fluorescence (Cal+), floc yields and nitrite did not accumulate in the medium. The role of nitrite reduction in flocculation is not well understood and deserves more attention. On the other hand, from these and other results (not reported here), EPS production appears to play an important role in cell aggregation during flocculation and pellicle formation. We expect that the use of these (or similar) mutants in root colonization studies should help our better understanding of associative symbiosis.

E-96

Nucleotide sequence of an Azospirillum nod homologous region.

M. Delledonne*, R. Porcari, C. Fogher. Istituto di Genetica, Università Cattolica, Piacenza 29100, Italy.

A 12 kb DNA segment of the A. brasilense Sp7 genome, that shows homology with an heterologous R. meliloti nod region, has been cloned (1) and partially sequenced. The construction of the physical map of the cosmid with this fragment and the DNA sequence analysis of some subclones has allowed the positioning and the identification of homology with nodG gene from R. meliloti. The nucleotide sequence of the putative nodG gene of A. brasilense shares 59% homology with nucleotide sequence of the nodG gene of R. meliloti (2). The predicted amino acid sequence of the nodG gene product has been compared with the protein sequence data base and results are discussed. Sequence analysis of this and others adjacent ORFs, apparently, give not insight on promoters consensus; this is consistent with others observations (3,4).

1. Antonelli et al. 1988. Abstr. XXXI Conv. Soc. Ital. Gen. Agr., Como, pp.59-60.
2. Debellé F., Sharma S.B. 1986. Nucleic Acid Res. 14:7453.
3. Bozouklian H., Elmerich C. 1986. Biochimie 68:1181.
4. Civardi L. et al. 1990. Abstr. this congress.

E-97

Isolation and characterization of regulatory sequences of Azospirillum brasilense.

L. Civardi, M. Delledonne, C. Fogher*. Istituto di Genetica, Università Cattolica, Piacenza 29100, Italy

Bacteria of the genus Azospirillum are diazotrophs associated with the roots of grasses. Considerable progress towards understanding the molecolar biology of their association with plants is based on the identification of plant-inducible genes. With this goal, we have isolated and sequenced a set of "costitutive" promoter-containing DNA fragments of Azospirillum brasilense Sp7 (1). Transcriptional fusions to CAT reporter gene of plasmid pKK232-8 (2) allowed us to identified in E. coli-costitutive promoters at the frequence of 3.4%. The first 17 sequences, isolated at 20 µg/ml Cm, have been classified in seven MIC classes of resistance from 20 to 280 µg/ml of Cm. One fragment from each class has been sequenced and the general features are argue in terms of regulation. Preliminary data on identification of root extract-plant inducible promoters are reported.

1. Civardi L., Fogher C. 1989. XXXIII Conv. Soc. Ital. Gen. Agr., 23-26 Oct., Alghero, pp.126-127.
2. Brosius J. 1984. Gene, 27:151-160.

E-98

Azospirillum brasilense **Sp7: p90 carries essential functions.**

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Azospirillum is a growth-promoting diazotroph which associates with roots of grasses. *Azospirillum* harbors large plasmids. Homology was previously found between DNA fragments containing *Rhizobium meliloti* nodulation genes and the 90 MDa plasmid, p90, of several *Azospirillum* strains. Two *A. brasilense* DNA fragments that correct mutations in the *R. meliloti* exopolysaccharide synthesis genes, *exoB* and *exoC*, were also shown to originate from p90. A more detailed characterization of the plasmid was undertaken to establish its physical map and to localize the *nod* homology and other regions.

Six loci were mapped, the region homologous to the nodulation genes, *nodPQ*, of *R. meliloti*, the *exoB* and *exoC* mutation-correcting loci, two loci for ampicillin resistance, and a region necessary for the maintenance of p90 as an independent replicon and which might correspond to the origin of replication. Self-transfer of p90 was not demonstrated but mobilization in *A. tumefaciens* of p90-Tn*5* was obtained at a frequency of 10^{-4}, with the helper plasmid pJB3JI. Fragments of p90 hybridized with plasmids of 90 MDa present in most strains of *A. brasilense* and some *A. lipoferum*, suggesting the existence of common functions on these plasmids and a plasmid family in *Azospirillum*. Several methods were used in attempts to obtain p90-cured strains, without success. However incompatibility with plasmids carrying p90 replication origin led to the obtention of derivatives apparently cured of p90. Further analysis revealed that p90 was integrated into the chromosome. p90 is therefore impossible to cure, suggesting existence of esssential bacterial functions on this plasmid.

E-99

Immobilization of Azospirillum spp in alginate beads as tool for cyst-formation. J.Ueckert[2]*, M.P. Stephan[3], J. Döbereiner[3], E.G. Niemann[2] and I. Fendrik[2]. [2]Inst. of Biophysics, Hannover FRG and [3]EMBRAPA-CNPBS-RJ./Brazil

The process of flocculation and cyst formation has been studied in Azospirillum spp. in batch culture. C-forms (cysts) have also been found in the root interior of Kallar grass and in the association of the strain sp_7 with tissue culture of sugar cane. It remains unclear if those C-forms are important for infection and association of the plant with the bacteria. A new approach to induce the formation of C-forms of A. brasilense, strains sp_7 and 245, was the entrapment of this microrganism in calcium-alginate beads. Those beads were produced by dripping 0.5g of cells in 100 ml solution of anionic polymer sodium alginate into a solution containing the multivalent cation Ca^{2+}. In continuous culture of Azospirillum - alginate beads the strains sp_7 and 245 form an agglomerate of completely round C-forms. These results demonstrate the utility of the alginate beads as a tool to elucidate whether cysts are resting structures or metabolic active stages. The present work will compare this species with other nitrogen fixing genera.

E-100

EXCRETION OF AMMONIA BY *Azospirillum brasilense*. H.B. Machado, S. Funayama, L.U. Rigo and F.O. Pedrosa*. Dept. of Biochemistry, Universidade Federal do Paranã, C. Postal 19046, 81504 Curitiba, Paranã, BRAZIL

Fifteen spontaneous mutants of *Azospirillum brasilense* strain FP2 (= Sp& Nal^r Sm^r) resistant to ethylenediamine derepressed nitrogenase in the presence of high concentration of NH_4^+ (10 mM) and half of them excreted substantial levels of NH_4^+ in N-free semi-solid medium. NH_4^+ excretion was abolished when argon replaced N_2 in the atmosphere. These mutants were found to be prototrophs for glutamine. Three of them showed high levels of constitutively adenylylated glutamine sinthetase, and nitrogenase activities partially resistant to NH_4^+ switch-off. These mutants were deficient (25-40%) in NH_4^+-uptake. Transconjugants containing plasmid pGE10 (*glnA ntrBC*) partially recovered NH_4^+-uptake and maintained their capacities to fix nitrogen in the presence of NH_4^+. Experiments relating the effect of these mutants on growth and N-assimilation by wheat and sorghum plants will be presented.

Supported by the Brazilian Research Council (CNPq).

E-101

Occurrence of Cellulolytic Enzymes in Azoarcus spp.

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Microaerobically N_2-fixing, gram negative rods originating from roots of Kallar grass (Leptochloa fusca (L.) Kunth) have been proposed to belong to a new genus Azoarcus spp. (see Reinhold-Hurek et al., this volume). Since some strains are occurring in high numbers in the endorhizosphere of field-grown Kallar grass, and strain BH72 has been shown to invade roots of gnotobiotically grown plants, we were interested if plant-polymer degrading enzymes are present. In bacterial colonies, β-glucosidase and cellubiohydrolase like activities were detected by fluorogenic substrates, and endoglucanase like activities by congo red staining of carboxymethylcellulose (CMC), indicating the presence of all three classes of cellulases in strains BH72 and SWub3, and of the first two in strains VB32 and S5b2. Ethanol in the growth medium enhanced or induced cellubiohydrolase and endoglucanase activity;glucose had no inhibitory effect. D-Glucose, D-cellubiose, CMC, and Avicell cellulose did not support growth of strain BH72. All three enzyme activities were mainly associated with cell surface fractions. Genes of BH72 for all three activities have been cloned and expressed in E. coli, those for β-glucosidase and cellubiohydrolase being linked. Both activities showed also the same mobility in isoelectric focusing gels, indicating the presence of only one enzyme, whereas two different bands of endoglucanase activity were detected. Furtner analysis of the genes will be presented.

E-102

The N- and C-terminal portions of NifV are encoded by two different genes in Clostridium pasteurianum. S.-Z. Wang*, D. R. Dean, J.-S. Chen, and J. L. Johnson. Dept. of Anaerobic Microbiology, VPI & SU, Blacksburg, VA 24061, USA.

A 7.44 kb DNA fragment beginning from the nifN-B region of C. pasteurianum (Cp) was cloned into pUC18. The cloned fragment contains 6 open reading frames (ORFs) beyond nifN-B. Regions resembling the putative Cp nif consensus promoter and upstream sequences were found only between ORFs 1 and 2. ORFs 2 through 5 probably constitute one transcriptional unit, and they have coding capacities for 285, 352, 268, and 274 amino acid residues, respectively. The predicted amino acid sequences of both ORFs 3 and 4 have regions showing a similarity to different regions of NifV of Azotobacter vinelandii and Klebsiella pneumoniae. These conserved regions were found (i) between the C-terminal 195 residues of ORF 3 and the C-terminal portion of NifV, and (ii) between the entire ORF 4 (268 residues) and the N-terminal portion of NifV. ORFs 3 and 4 are therefore designated nifVω and nifVα, respectively. Plasmids containing nifVα and nifVω, singly and in combination, were used to transform an A. vinelandii strain (DJ388) having nifV deleted. The nifV phenotype of the A. vinelandii mutant was rescued only when both Cp nifVα and nifVω were introduced into the mutant. The results suggest that NifV contains two or more essential domains which can involve either one or two polypeptides.

E-103

Construction of a genomic library from nitogen-fixer Alcaligenes faecalis. C.B.You, W.L.Hai, H.Wang, C.Zhang and B.Wang* Inst.Application of Atomic Energy, CAAS. *Inst.Genetics, Acad.Sin. P.O.Box 5109, Beijing 100094 P.R.China

6 strains of A.faecalis(Af) have been isolated and identified. Strain A15H which possesses rather high nitrogenase activity was selected fro constructing of genomic library. The chromosomal DNA was partialy digested with Sau 3A1. Fragments in range of 13-20 kb wewre selected. These resulting fragments insert in a bacteriophage $EMBL_4$ vector. The probability of finding a unique A. faecalis chromosomal sequence amongest these colonies is more than 99%. The transduced colonies N are 2.5×10^5 pfu. By using pSA30 and pMC 71A plasmids as probes, which contain nif KDH and nif A respectively, we were sure that we had a library of Af DNA. The subclone of nifH also has been preparing. The results also imply that there may be more than one copy of the nif sequence in A. faecalis.

E-104

Simultaneous occurrence of Photosynthesis and Nitrogen fixation in *Gloeocapsa* SBS CY11**, R.S. Shanthakumar Hopper* and S. Shanmugasundaram, Dept. of Microbiology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India.

In the absence of spatial separation of Photosynthesis and Nitrogen fixation, the wild type *Gloeocapsa decorticans* temporally separates these two activities by performing Photosynthesis in light and Nitrogen fixation in dark. However a mutant isolated by NTG treatment *Gloeocapsa* SBS CY11 performs both these activities in light. Infact the activity of Nitrogen fixation measured as acetylene reduction is 4-5 fold higher in light compared to the activity in dark. Also 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at 10^{-4} M and 10^{-5} M in the presence of light instead of enhancing the acetylene reduction inhibits the activity demonstrating that the Nitrogenase activity in this mutant is PSII dependent.

**Property of the Department of Microbiology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India.

E-105

Complementation of nitrogen fixation genes in the cyanobacterium *Anabaena* sp. PCC 7120: Identification of a gene controlling heterocyst differentiation.

W. J. Buikema* and R. Haselkorn. Dept. of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA.

Anabaena 7120 is a filamentous cyanobacterium that produces specialized cells called heterocysts at regular intervals when deprived of fixed nitrogen under aerobic conditions. Heterocysts are anaerobic compartments for nitrogen fixation in which many structural and physiological changes have occurred. We have isolated 140 mutants of *Anabaena* 7120 deficient in the ability to grow aerobically on media lacking fixed nitrogen. Eight of these mutants representing several phenotypes were complemented by conjugation with a cosmid library of wild-type *Anabaena* 7120 DNA. Three mutants representing two phenotypes were found to be complemented by overlapping cosmids. One of these mutants, 2-16, fails to differentiate heterocysts under all growth conditions. A single 9.0 kb *Nhe*I subclone of one of the complementing cosmids can complement each of the three mutants. A 2.4 kb fragment that complements mutant 2-16 contains an open reading frame (ORF) capable of encoding a 299 amino acid protein. This ORF is transcribed at a low level under nitrogen replete conditions but at a much higher level within 6 hours of transfer to N^- conditions. Conjugation of deletion subclones of this region into 2-16 showed that this ORF is both necessary and sufficient for complementation of the Het^- phenotype. The mutation in 2-16 converts a serine in the wild type ORF into an asparagine. Both 2-16 and wild-type cells containing the intact ORF on a plasmid display an increased frequency of heterocysts. These results suggest that this gene, which we have named *hetR*, encodes a product that is involved in the regulation of heterocyst differentiation.

E-106

Independent regulation of *nifHDK* operon rearrangement and transcription during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120.

J.W. Golden*, L.L. Whorff, and D.R. Wiest. Department of Biology, Texas A&M University, College Station, Texas 77843-3258, USA.

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 produces terminally differentiated cells called heterocysts that are highly specialized morphologically and biochemically for nitrogen fixation. The genome of *Anabaena* 7120 undergoes at least two specific developmentally regulated DNA rearrangements during heterocyst differentiation. One rearrangement involves the nitrogen-fixation operon *nifHDK*. During heterocyst differentiation, an 11-kb DNA element is excised from the *nifD* gene by site-specific recombination. The excision results in the formation of the complete *nifD* coding sequence and allows expression of the three genes from a single promoter. The *xisA* gene, located on the 11-kb element, is required for the excision of the element and presumably encodes a site-specific recombinase. Transcription and rearrangement of the *nifHDK* operon both occur late during heterocyst differentiation, about 12-18 hours after induction. We show that these two events are independent of one another. Northern analysis of the *xisA* mutant strain DW12-2.2, which can not excise the *nifD* 11-kb element or fix nitrogen, showed that the *nifH* and *nifD* genes are transcribed on the unrearranged chromosome after heterocysts are formed. Therefore, the activation of the *nifH* promoter in heterocysts is not dependent on rearrangement of the operon. The *nifK* gene was not transcribed in DW12-2.2, indicating that its expression is dependent on the *nifH* promoter and excision of the 11-kb element from the operon. To test the hypothesis that transcription from the *nifH* promoter induces excision of the 11-kb element, we deleted a 1.85-kb fragment containing the *nifH* promoter from the chromosome to produce the mutant strain LW1. LW1 formed heterocysts, but did not fix nitrogen and showed no transcription through *nifD*. Southern analysis of LW1 showed normal rearrangement of the 11-kb element from the *nifHDK* operon, indicating that transcription from the *nifH* promoter is not required for developmentally regulated excision of the 11-kb element.

E-107

An open reading frame in the *Anabaena* 7120 *nifD* excision element exhibits homology to cytochromes P-450. P.J. Lammers*, S. McLaughlin, S. Papin, C. Provencio, A.J. Ryncarz. Dept. of Chemistry, New Mexico State University, Las Cruces, N.M. 88003, USA.

Many diazotrophic cyanobacteria undergo chromosomal rearrangments during heterocyst differentiation. The *nifD* gene is often split by a large DNA element, the excision of which is necessary for nitrogenase synthesis but not for heterocyst formation. Strains that have lost the *nifD* element show no obvious differences in heterocyst development, spacing, or function. Thus, any selective advantage associated with the presence of the element must be subtle, or unrelated to nitrogen fixation.

The determination of 5000 base pairs of DNA sequence extending from the right border within the *nifD* element in *Anabaena* 7120 has been completed. Several open reading frames have been found, one of which encodes a predicted polypeptide of about 40,000 daltons. Significant homology exists between this sequence and patterns diagnostic of six subfamilies within the cytochrome P-450 monooxygenase superfamily. The homology extends over most of the polypeptide, and includes a conserved cysteine that acts as a fifth ligand for the heme iron in the P-450 proteins.

Transcription through the region of the open reading frames has been examined. RNA was isolated from whole filaments aerobically induced in continuous light. Probes specific for the P-450 homologous sequence and one other ORF identify very low but finite levels of RNA isolated 24, 30, and 36 hours after induction. These signals were highest at the 30 hour time point. Control *nifD* transcript levels were many fold higher and increase at each time point. Little or no signal is produced by these probes with RNA isolated from vegetative cells.

We speculate that the P450-like sequence may confer the ability to degrade exogenous inhibitory molecules or initiate utilization of unusual organic nitrogen sources thruough N-dealkylation or oxidative deamination reactions. Alternatively, the protein may serve as an oxygen carrier to a coupled or uncoupled respiratory oxidase functioning in heterocysts.

E-108

Genetic Variation among Edaphic Species of the N_2-Fixing Cyanobacterium Nostoc. W.J. Zimmerman and D.E. Culley. Dept. of Agronomy & Soils, Washington State University., Pullman, WA 99164, USA.

Diversity among soil-inhabiting cyanobacterial species of the heterocystous genus Nostoc (N. punctiforme and others) was evaluated. Three cultured isolates from facultative symbioses with higher plants--Gunnera (2) and Encephalartos (1)--and three naturally asymbiotic isolates were examined. Little similarity was found among the cyanobacteria, even isolates of N. punctiforme, from DNA hybridizations with the genes coding for phycocyanin (cpcA & B), allophycocyanin (apcA & B), gas vesicle protein (gvpA1), and part of the nitrogenase complex (nifH). One gene (gvpC) was not found in any strain, distinguishing this group from taxa which show homology. The latter cyanobacteria are able to generate abundant gas vesicles rapidly and have >1 copy of gvpA1. Incubation with 16 methylation sensitive restriction endonucleases indicated a high degree of methylation. Results from cpcA & B hybridizations showed that dcm-like methylase was not present in all strains. The genetic variation within Nostoc was judged to reflect different soil origins, regardless of species or symbiotic history.

E-109

Development of mobilisable vectors for the cyanobacterium Plectonema boryanum. Rakesh Tuli, Ajay K. Vachhani and R.K. Iyer. Molecular Biology and Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, INDIA.

Nitrogen fixing cyanobacteria are not amenable to conventional methods of gene transfer. Mobilisable plasmids constructed by utilising the Nostoc replicon are suitable for a narrow range of heterocystous cyanobacteria. In order to develop a mobilisable vector system for the nonheterocystous cyanobacterium Plectonema boryanum UTEX 596, we have characterised its extrachromosomal DNA. One of the plasmid replicons seems to be functional in Escherichia coli. A 1.5 kb plasmid is present as 3-4 copies per genome in the cyanobacterium. A small proportion of the plasmid is present as multimers. The small plasmid shows no homology to the other two bigger plasmids. A variety of conjugal plasmids including RP4, R64 drd 11 and pRK 2073 could mediate the transfer of a broad host range plasmid from E. coli to P. boryanum Results on the mobilisable vector, use of several resistance markers and possibilities in transposon mutagenesis in the cyanobacterium would be discussed.

E-110

MORPHOLOGY AND LIFE CYCLE OF THE NITROGEN-FIXING CYANOBIONT IN THE SEVEN AZOLLA SPECIES.

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The taxonomic status of the cyanobacteria present in Azolla cavities has been questioned for a long time. Synchronous morphological differentiation in vegetative cells and in heterocysts frequency occurs between the apex and the older leaves in younger plants. In the apex, trichomes are composed entirely of small vegetative cells (hormogonia). In older leaves cyanobacteria are enclosed in a cavity and embedded in a mucilaginous material, trichomes are straight to curved and heterocysts are intercalary and terminal. Akinetes when present are between vegetative cells, adjacent to one or both sides of the heterocysts, single or in chains with variable shapes and sizes depending on the Azolla species. Akinete germination gives rise to a hormogonium which can form adjacent to or between two heterocysts. To the best of our knowledge this is the first report of occurrence of multiple heterocysts in the trichomes of the cyanobacteria in all Azolla species studied. Plant chromatic changes and light intensity regulate the life cycle and pigment composition of the cyanobacteria. Based on microphotographic analysis and the new concepts of classification, the cyanobionts in Azolla are more appropriately classified in the genus Nostoc rather than Anabaena.

SESSION F

Nitrogen fixation in agriculture and industry
F-01 - F-40

F-01 Nitrogen Fixation by *Phaseolus vulgaris* L. in response to inoculation. B. Ahmed* and R.H. Phelps. CARDI, UWI, Trinidad, W.I. Present address: Consultant, Mona, Kingston, Jamaica and Caroni Research Station, Waterloo Road, Trinidad.

Two bean cultivars from CIAT, were evaluated in the greenhouse on two acid soils of Trinidad, for their shoot weight, Nodule weight, Total N and Nitrogenase activity. Uninoculated controls, with or without N, nodulated and showed nitrogenase activity in the soils, indicating presence of viable *Rhizobium phaseoli*.

Inoculation by differing strains resulted in significantly higher nodule weight, Total N and nitrogenase activity compared to controls. A local strain, isolated from an acid, infertile soil, increased nodule weight, Total N and nitrogenase activity of plants. It would appear that local isolates can supply adequate nitrogen to the bean plants.

F-02 Nodulation and nitrogen-fixation improvement in soybean cover-inoculated with *Bradyrhizobium japonicum* at the three-node stage. G. Ciafardini* L. Busi. G. Marinelli and L.M.R. Arena. Soil Microbiology Laboratory, Istituto Sperimentale per le Colture Industriali, Via di Corticella n.133, 40129-Bologna, Italy.

In clay soils with low populations of *Bradyrhizobium japonicum*, seed inoculation of soybean (*Glycine max* (L.) Merr.) often results in a small number of nodules distributed mainly on the primary root, due to poor soil mobility of the inoculated symbiont. To improve nodulation on secondary and adventitious roots, we cover-inoculated soybean at the three-node stage (V_3) with B. *japonicum* using 150 m^3 of irrigation water in a silty clay loam soil (vertic xerothent). Compared to traditional seed-inoculation alone, seed plus cover-inoculation at V_3 doubled nodule biomass and also increased nitrogen fixation, plant nitrogen concentration seed protein (6-7%) and yield (10-12%) in the Hodgson soybean cultivar grown as a primary crop. Seed- and cover-inoculation of this cultivar grown as a secondary crop tripled nodule biomass and increased nitrogen fixation and plant nitrogen concentration, but in some case not seed yield.

F-03

ADAPTATION OF EFFICIENT Bradyrhizobium japonicum STRAIN FOR INOCULATION OF SOYBEANS IN CERRADO SOILS

M.C.P., Neves*; G.R., Botelho; M.L.G., Ramos; CENTRO NACIONAL DE PESQUISA EM BIOLOGIA DO SOLO, EMBRAPA, 23851 - Seropédica - RIO DE JANEIRO - BRAZIL

Substitution, by strain CB1809, of the strains currently used as commercial inoculant for soybeans in Brazil and neighbouring countries may increase seed yields in about 30%. This is due to higher nodule efficiency and improved transfer of nitrogen to the grain (Neves et al 1985, J. exp.Bot.36:1179-1192). The utilization of this efficient strain is however limited by its inability to survive the prevailing soil conditions of the major soybean producing area, the Cerrados (Brazilian edaphic savanahs) or to compete with rhizobia already established in cultivated soils. Seeds inoculated with strain CB 1809 were sown in a cerrado soil never cropped before. The few nodules formed were tested individually for high ARA and lack of H_2 evolution, selecting the most active nodules for bacteria identification, and used to inoculate the following crop. By repeating this procedure it was possible to improve the nodulation ability of some isolates as compared with the original strain. The adaptation increased antibiotic resistance, reduced exopolyssacharide production but did not alter the efficiency of the isolates.

In soil containing an established population of rhizobia, however, the isolates showed no advantage over the original strain, indicating that adaptation increased survival but not competitivity.

Selection and adaptation in soil containing an established population of rhizobium is now in process and show promising results.

F-04

Response of Soybean to Bradyrhizobium japonicum strains in field trial.

M.M.Rahman* and C.L.Sanoria, Dept. of Soil Sci. & Agri. Chemistry, Banaras Hindu University, Varanasi-221005, INDIA.

A field experiment was conducted in the Inceptisol of Varanasi following randomized block design to see the response of soybean cv PK 327 to different Bradyrhizobium japonicum strains. The soil was clay loam in texture having pH 8.1, organic matter 1.37% total N 0.05% and available P 14.66 ppm. Phosphorus @ 20 kg/ha and Mo @ 20g/ha were used as basal dressing. The strains used for inoculation were TAL-94, TAL-377, USDA-110, TSH-501, SB-16, S-646, PNT-103, BAU-107 and BAU-118. Data on nodulation, growth characteristics and grain yield of soybean as influenced by different strains were recorded. Soybean responded differently to strains and the highest grain yield of 24.2 q/ha was obtained with the strain USDA-110. The strain BAU-118 and S-646 produced the highest nodule mass of 175 and 598 mg/plant at 50 and 72 DAP, respectively. Nodule masses of uninoculated control were 0 and 30 mg/plant at 50 and 72 DAP, respectively. Ranking of strains for total above ground dry matter accumulation at 72 DAP was S-646 > TSH-501 > BAU-107 > BAU-118 > USDA-110 > PNT-103 > TAL-377 > TAL-94 > Control > SB-16.

F-05 Influence of strain and variety on yield and biological nitrogen fixation by bean (Phaseolus vulgaris).

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In pot experiment influence of strain Rhizobium phaseoli (2) and varieties (2) of bean Phaseolus vulgaris on yield and biological nitrogen fixation was investigated. Dry matter, content of nitrogen (N) and ^{45}N in all parts of plant was determinated. Barley was used as non-fixing plant. Significant differences in yield of seed caused by interaction of strain x variety was observed. Amount of fixed nitrogen was significanttly different among strains. Correlation between amount of fixed nitrogen and harvesting index was calculated.

F-06 Interaction between Rhizobium leguminosarum strains and varieties of fababean (Vicia faba minor) on yield and biological nitrogen fixation

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In pot experiment biological nitrogen fixation using (^{15}N) and C_2H_2 methods in diferent stages of fababean was investigated. Three varieties and 4 strains of Rhizobium leguminosarum were used. Dry matter, contents of nitrogen (N) and ^{15}N was determinated in all parts of plant. Summer wheat was used as non-fixing plant. Significant diffrences in yield of seed, amount of fixed nitrogen for varieties and strains as well as for interaction variety x strain was estimated. Chemical analysis showed differences in location of ^{15}N in different part of plant. Liniar regression between amount of fixed nitrogen and reduction of C_2H_2 was calculated. Harvesting index has been changed as results of interaction between variety x strain.

F-07 Regularities of Dynamics of Nitrogenase Activities in the Root Zone of Plants with Different Genotypes.

V.T.Emtsev, Dept. of Microbiology, the Timirjazev Agricultural Academy, I27550 Moscow, USSR

The comparative characteristic of dynamics of nitrogenase activities in the root zone of fifty nine varieties of rice, wheat, oats, barley, corn and millets is given. It is found out that the level of nitrogenase activities in the root zone of this or that variety is determined by a genotype of a given plant. Among the varieties of studied plant species some representatives with high actual and potential nitrogenase activities are found. We found out a direct dependence between dinamics of photosynthesis intensity of a certain plant variety and dynamics of nitrogenase activities in its root zone. An ATP (adenosine triphosphate) content in a rhizosphere of different varieties of non-legume plants was determined. It was found out that the highest amount of ATP is in the rhizosphere of the plant varieties that have high photosynthetic activity (especially varieties of rice and C_4-plants), while varieties with low photosynthetic activity have the least ATP content in the rhizosphere. It was mathematically prowed the pluralistic correlative dependence between the intensity of photosynthesis, number of diazotrophes, ATP content and nitrogenase activities in the root zone of the plants of definite varieties.

F-08 Genotype non-legume plants and associative N_2-fixation.

V.T.Emtsev, M.I.Chumakov, Dept. of Microbiology, the Timirjazev Agricultural Academy, I27550 Moscow, USSR

The associative nitrogen fixation activity (nis) of 30 cultivars with different genomes of Triticum L. was investigated. Nis-ability depended of genome ploidy ($r=0.9724$). The highest activity among 9 tested wheat species was detected in Triticum aestivum. The old selection varieties (Rusak, Poltavka) with hexaploid chromosome type posses the maximum nitrogenase activity. The differencies between spring and winter wheat cultivare groups are not singnificant (at $P<0.0I$). Screening of 2I monosomic lines of wheat Saratovskaya 46 (ABD genome) for nis-ability showed the trait inheritance to have polygenic character. The following row $A>D>B$ can be made up according to the degree of different genomes effect on N_2-fixation activity. Individual plants incapable of supporting N_2-fixation in soil bacteria population ("0-plants") were noted within the same wheat cultivar.

F-09

Stimulation of plant growth by inoculation with nitrogenase derepressed mutants of phototrophic bacteria. S.Shestakov*, L.Mikheeva, V.Groshev, V.Frolova, N.Belavina, T.Mitronova. Dept. of Genetics, Moscow State University, Moscow II7809, USSR.

A number of derepressed for nitrogenase drn-mutants of photosynthetic purple bacterium Rhodobacter sphaeroides and cyanobacteria Anabaena azollae and Anabaena var I8IB have been obtained by various methods of selection. These drn-mutants have a low level of glutamine synthetase and excrete ammonia /produced by nitrogen fixation/ into the medium. The influence of inoculation with drn-mutants on the development and growth of several varieties of non-legume plants have been studied in microvegetative experiments. R.sphaeroides drn-mutants were revealed which significantly stimulate the development of roots and stems of rape and tomato. Some Anabaena drn-mutants increase /by 40-80 per cent/ the seedling growth of certain varieties of rice, watercress and tomato. Some of these mutants produce and secrete biologically active compounds the nature of which is being analysed now. The most productive partner systems were used for studying the contribution of excreted ammonia and bioactive compounds into stimulation effects. Such associative systems with nitrogen-fixing phototrophic bacteria are suitable models for the investigation of biochemical mechanisms and genetic control of plant-bacteria interactions.

F-10

Improved grain protein content in Argentine crops of *Phaseolus vulgaris* through inoculation. J.C. Pacheco Basurco[1], A. Perticari[2], A. Gauna[1], R. Diéguez[1], A. Pellegrini[2], P. Figueroa[2], J.L. Boiardi[3], E. Arrarás[3], A. Lodeiro[4], and G. Favelukes[4]*. [1]Microbiol. INTA; [2]EEA INTA, Salta; [3]CINDEFI, La Plata; [4]QBI, UNLP, La Plata, Argentina

Phaseolus beans (originated from Central and South America) provide low cost, essential protein nutrients to large populations throughout Latin America. Both N-fixation and grain yield in bean crops in Latin America are generally very poor. Bean cultures in NW Argentina (a center of origin of the primitive *P. vulgaris* var. aborigineus) are spontaneously but poorly nodulated, and their moderate grain yields do not generally show a positive response to *Rhizobium* inoculation. Local soils, with acceptable populations of symbiotically able native rhizobia, support abundant 'spontaneous' nodulation in the laboratory and greenhouse; limitations to field nodulation appear to arise from stress due to high temperature combined with low humidity in the soil. Here we report a beneficial effect of inoculation in field trials, whereby the protein-N content in grain from inoculated plots has been consistently 7 to 15% higher, compared to non-inoculated controls. Even in the absence of yield increases, this significant improvement in the nutritional quality of grain would by itself warrant the systematic use of inoculants in bean cropping.

F-11

Influence of *Rhizobium* inoculation on food legume production in Bangladesh. M.S. Hoque, Department of Soil Science, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Increasing attention is now being paid in Bangladesh to the use of *Rhizobium* inoculation as a means of reducing dependence on costly urea fertilizer in food legume production. A considerable amount of works have been carried out by the author on the response to *Rhizobium* inoculation by several legume crops and on the effectiveness of different strains of rhizobia. Results of field trials showed distinct beneficial effects of *Rhizobium* inoculation on nodule formation, shoot dry matter, shoot N content and yield of groundnut, mungbean, soybean, blackgram, lentil and grasspea. In most cases, the increases in yield of the crops due to inoculation were much higher than that observed in urea-N treated plots. Several *Bradyrhizobium japonicum* strains designated as BAU 101, BAU 107, BAU 118 and TAL 102 have been found highly effective on soybean giving as high as 100-200% yield increase over uninoculated control. For groundnut, the strains BAU 700, BAU 702 and TAL 1371 were found promising producing 25-70% higher nut yield over control. The strains BAU 604 and BAU 606 were found to give 24-47% higher yield of mungbean over control. In case of soybean, response to *Rhizobium* inoculation was highly remarkable because of the absence of soybean rhizobia in the soils.

F-12

Selection of Suitable carriers for rhizobia incubating under different temperatures. S.H.Salem * and S.A.Z. Mahmoud. Dept. of Agric.Botany, Fac. of Agric., Zagazig Univ. and Microbiology Dept. Ain Shams Univ., Egypt.

Nine carriers were tested, in laboratory under sterile conditions, for the suitability of *Rhizobium leguminosarum*. The used carriers were, silt clay loam soil, sandy soil, saw dust, talcum powder, charcoal and compost either alone or mixture of them. The cheap price of such materials without using additives of nutrients was of great importance in chosing the tested carriers.The main goal of these laboratory experiments is to recognize the suitable carrier for maintaining rhizobia for longer period viable under room & refrigerator temperatures. Counts of rhizobia were determined periodically under aseptic conditions every 15 days up to 120 days. Results have shown that the rhizobia grew well and increased in their numbers in almost 7 carriers up to 30-45 days, The numbers were decreased thereafter, with maintaining considerable numbers of viable rhizobia at the end of the experimental period (3-4 months). Using saw dust and talcum powder as carriers either alone or mixture with clay soil, the numbers of rhizobia were rapidly decreased in short periods.The growth of rhizobia have shown better results with longer periods of viability (3 months) when grown on mixture of silt clay loam soil + charcoal + compost (5:3:2) and incubated on room temperature. Therefore the latter could be used safely as a carrier for rhizobia for the farmer use within 3 months from the production.

F-13 Influence of Benlate on the growth and nitrogen fixation in three species of **Azolla**.

S. Kannaiyan, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.

Azolla is an aquatic water fern that fixes atmospheric nitrogen in symbiotic association with a cyanobacterium - **Anabaena azollae**. **Azolla** fronds are commonly affected by a soil - borne pathogen - **Rhizoctonia solani**. Benlate - a systemic fungicide was used for treating three species of **Azolla** with a view to assess its effect on growth, chlorophyll synthesis and nitrogen fixation potential besides checking the **R. solani** infection. Benlate at 2.5 ppm ai has induced the growth of **A. microphylla** - BR-GI, **A. caroliniana** - WT-V and **Azolla** sp - ST-SI. The growth rate induction in **Azolla** sp - ST-SI was significant due to Benlate treatment. The culture **Azolla** sp - ST-SI has recorded maximum nitrogenase activity followed by **A. microphylla** - BR-GI due to Benlate treatment. Among the three species of **Azolla** tested **A. caroliniana** - WTV and **Azolla** sp - ST-SI have increased the total chlorophyll content significantly due to Benlate treatment. Benlate treatment reduced the nitrogen content in **A. microphylla** - BR-GI and **Azolla** sp - ST-SI while carbon content was not affected much.

F-14 Classification of Azolla layers and approximation of biomass by Visual diagnosis.

M.H.Mian. Deptt. of Soil Science, Bangladesh Agricultural University, Mymensingh.

A set of criteria has been fixed to classify layers of Azolla by Visual observations. Five types of layers have been suggested: (a) No layer (NL).i.e. no end to end cover of the area; (b) loosely covered layer (LCL) i.e. end to end coverage leaving 25% of the area uncovered as inter-frond spaces; (c) moderately covered (MCL) i.e. as in (b) but still 10% of total area remained uncovered as inter-frond spaces; (d) well covered layer (WCL)i.e. a thick cover with less than 2% empty inter-frond spaces; and (e) densely covered layer (DCL) i.e. a compact mat with no space visible. The fresh biomass of LCL, MCL, WCL and DCL were 7-8, 10-12, 14-15, and 16-18 ton/ha; the corresponding N contents were 12-15, 20-21, 32-35 and 40-45 kg ha^{-1}, respectively. The formation of LCL, MCL, WCL and DCL took 4,6,8 and 10 days for Azolla caroliniana Wild and 8,10, 12, and 14 days, respectively for Azolla pinnata R.Br. if both applied at the rate of 0.2 kg $\bar{m}^2$. In addition, spontaneous generation of new frond has been studied.

F-15 Organic matter amendment enhanced nodulation and growth of multi-purpose legume trees.

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Composted sugar mill filter cake and/or mill ash were shown to enhance seedling nodulation and growth of certain legume trees. Grassland soil with pH 5.6, 1.0% OM, and 3.0 ppm P when used as potting medium could hardly support growth of 3-month old seedlings. However, application of 5.0 g ash to 400 g potting soil enhanced biomass production of Leucaena diversifolia, L. leucocephala and Calliandra callothyrsus by 106.04%, 321.4% and 743.0% respectively which statistically paralleled the effect of inorganic NPK. Ash was also found to be more effective than commercial 14-14-14 fertilizer in improving nodulation of the two Leucaena species.

A 6.0 g tableted organic formulation with 2.04%N, 4.60% P_2O_5 and 1.44% K_2O when supplied to potted Pterocarpus indicus and Acacia auriculaeformis produced an average of 46.8 and 97.6 nodules, respectively as compared to only 19.8 and 25.6 nodules from the unfertilized control. Similar effects on nodulation and growth were likewise exhibited on other legume trees.

Pilot testing of the tableted formulation under field condition in seven regions of the country is underway and is being financed by the Department of Environment and Natural Resources.

F-16 Effect of green manures on rice crop.

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Nepal is a predominantly an agricultural country where mineral fertilizer is very scarce and costly due to lack of mineral fertilizer plant. So only one cheaper alternative is to harnesh locally available green manures like Sesbania aculeata, Sesbania rostrata, Azolla pinnata and Vigna radiata (Moong beam). These green manures are fast growing and fix nitrogen in a tune of 40-100KgN/ha within 50-70 days period. These green manure species can be easily decomposed and increase rice grain yeild from 25-40%. These species can contain high nitrogen, 3.5-5.0% in their leaves, easy to propogate and also resist at adverse conditions and so their species serve as the best alternative to chemical fertilizer in the rice farming systems.

F-17 Late nitrogen fertilization effects on nodulation, N_2-fixation and yield in determinate and indeterminate soybeans (G. max L. Merr.).

F. Miceli*, M. Martin and G. Mosca. Ist. Produzione Vegetale, Udine University, P.le Kolbe 4, 33100 Udine (Italy).

A field experiment with nod+ soybean varieties and a nod- line, all inoculated with B. japonicum strain G49 (Lipha), was performed in Udine (N-E Italy) on a typic xerofluvent soil in 1987 and 1989. A standard indeterminate (ID) variety (Hodgson78) and a new determinate (DT) one (Spot), all of Maturity Group I, were tested in 1987, while in 1989 two couples of cultivars were used. A nod- line (L65) was always used as a non-fixing, reference crop. On these plants two mineral N treatments were imposed: none (N-0) and 150 kg N ha^{-1} applied at R4 growth stage (N150-L). The objectives were: i) to estimate the nitrogen amount deriving from N_2-fixation at the end of the crops; ii) to compare N_2-fixation (C_2H_2 reduction) and nodulation in late growth stages, together with seed yield of different growth habit soybeans. In 1987, about 60% and 42% of the biomass total nitrogen (BTN) at harvest came from N_2-fixation in N-0 and N150-L treatments; with the latter, only 10% of the BTN came from fertilizer. Total nodule mass per plant was depressed by N150-L, particularly in ID types. On R7 growth stage, nodule mass was about 35% lower in 1989 than in 1987. The specific ARA, performed on nodules of late growth stages, showed a higher functionality of DT variety nodules with N150-L treatment. ID varieties were able to take advantage of late N supply, performing higher seed yield, mainly because of their open node formation on the stem.

F-18 EFFECT OF AZOSPIRILLUM INOCULATION ON THE AMINO ACID CONTENT IN ROOTS AND SHOOTS OF WHEAT, BARLEY, PEAS AND LUPIN.

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This experiment was peformed to study effect of Azospirillum brasilense inoculation on the amino acid concentration in wheat, barley, peas and termis at 30, 60 and 90 days after planting. Cereals were inoculated with Azospirillum, legumes were inoculated with either Rhizobium or with Azospirillum or with a mixture of both inoculants, uninoculated plants were used as control. Amino acids were extracted & measured in both roots and shoots of plants. Results indicate that, the concentrations of amino acids were generally higher both in roots and shoots in all plants inoculated with Azospirillum inspite of the plant age. In legume tissues, the highest concentration of amino acids obtained in plants inoculated with a mixture of both inoculants, followed by Rhizobium, then Azospirillum. The differences in amino acid concentrations between Azospirillum inoculated and uninoculated treatments were more clear in the early stage of growth. There are essentially no qualitative differences in the known amino acids in the tissues of both roots and shoots whether inoculated or uninculated plants.

F-19 Increases of Nitrogen and Phosphorus in Gramineae Plants inoculated with *Azospirillum amazonense* Y1. S.D.Song*,.G.Park and K.B.Lee. Dept. of Biology, Kyungpook National University Taegu, Korea

We inoculated young corn and rice seedlings with the culture of *Azospirillum amazonense* Y1, a microaerobic and acid-tolerant nitrogen fixer, which grew associatively in roots and rhizosphere of Gramineae plants. During the growing period,the plant parts were analyzed for the inoculation effects on the growth and dry matter production and changes of nitrogen and phosphorus quantity . The plants treated with *Azospirillum amazonense Y1* in the rhizosphere showed increases in the growth of height, leaf area, and dry weight in comparison with untreated plants by 15.4%, 65.4%,and 33.7% , respectively, on the day of 56th after sowing. The treatment of the associative nitrogen fixing bacteria resulted in the increase of total nitrogen quantity of the higher plant by 38.0%. While the total phosphorus quantity in each organ of the plant showed two to three-fold accumulation as the control plant. The associative bacteria accelerated the growth of the under ground parts more than those of the upper ground parts of the plant, and caused decreases in T/R ratios.

F-20 **Inoculation by *Azospirillum brasilense* and Rhizobium ssp on *Cicer arietinum* (chick-pea).** Maddalena Del Gallo* and Paola Fabbri. ENEA, C.R.E.-Casaccia, Dept. of Agrobiotechnology, Via Anguillarese 301, I-00060 Rome, Italy.

According to several data reported on the literature, inoculations with rhizobacterium *Azospirillum* spp. seems to interact positively on the symbiosis *Rhizobium*-Legumes (Yahlom *et al.*, 1987, Can. J. Microbiol).
Two italian cultivar of chick pea "Calia" (spring cv.) and "Califfo" (winter cv.) were inoculated with *R. leguminosarum* bv *ciceri* USDA 3379 (from Mexico) and *Azospirillum brasilense* Cd in pots containig a mixture of sand and perlite (2:1 vol) in greenhouse. Controls were carried out in plants uninoculated, or inoculated with single strains. Plants were watered weekly with a Jensen N-free solution.
The results showed a positive statistically significant effect of *Azospirillum* on both varieties inoculated with *Rhizobium*. The highest increase was observed in the number of nodules per plants (about 34% in "Califfo" and 23% in "Calia") and in nodules dry weight (70% and 53% respectively) in comparison with plants inoculated with *Rhizobium* alone. Roots and shoots dry weight increased 26% and 46% in "Califfo", and 27% and 21% in "Calia". However, no effect was observed in the ARA activity: the nM of C_2H_4 reduced per plant were the same in plant inoculated with *Rhizobium* alone and with both *Rhizobium* and *Azospirillum*. The winter cultivar Califfo showed, in general, a better response to the inoculation.

F-21

Infection of sugar cane by Acetobacter diazotrophicus and other diazotrophs. V.M. Reis*, S. Urquiaga, M.A. de Paula and J. Döbereiner. EMBRAPA-CNPBS, Km 47, Seropédica, 23851, Rio de Janeiro, Brazil.

Recent N balance and ^{15}N isotope dilution experiments have confirmed that certain varieties of sugar cane are able to obtain substantial contributions from associated dinitrogen fixation. Furthermore, the newly discovered bacteria Acetobacter diazotrophicus has been identified as an endophyte of the roots, stems and leaves of sugar cane. Results of experiments with sterile micropropagates of sugar cane inoculated with this and other diazotrophs will be reported. Infection with A. diazotrophicus was observed either in vitro or after root tips had been cut but not when healthy plants were exposed to the inocula. Infected plants showed increased root and shoot production and higher tiller numbers. Data on the performance of such plants after transplanting to the field and on the interference of VA mycorrhizae will also reported.

F-22

Quantification of biological nitrogen fixation associated with 10 varieties of sugar cane. R.M. Boddey*, S. Urquiaga and J. Döbereiner. EMBRAPA-CNPBS, Km 47, seropédica, 23851, Rio de Janeiro, Brazil.

Ten varieties of sugar cane, comprised of 7 Brazilian commercial cultivars and 3 wild species, and a non-N_2-fixing control plant (Brachiaria arrecta) were planted in a reinforced concrete tank (20 x 6 x 0.8m) filled with a nitrogen deficient soil mixed with ^{15}N labelled organic matter. Some of the varieties (eg. CB 45-3 and SP 70-1143) maintained a stable productivity of more than 175 ton ha^{-1} over three consecutive annual harvests and the plants accumulated more than 220 kg N ha^{-1} at each harvest even though no nitrogen amendments were made. Both the N balance and ^{15}N isotope dilution data confirmed that some of the varieties could obtain large contributions from plant associated N_2 fixation.

F-23 The effect of Azospirillum brasilense on the root nodules formation, nitrogen fixation and productivity of leguminous plants. I.N.Andreeva*, T.V.Red'hina, K. Mandchan, S.F.Ismailov. K.A.Timiriazev Institute of Plant Physiology, Academy of Sciences of the USSR, Moscow, 127276, USSR. Institute of Microbiology, Academy of Sciences of the USSR, Moscow, 117811, USSR

The combined inoculation of kidney bean and of soybean seeds with the appropriate strains of Rhizobia and Az.brasilense results in the increase of the quantity and weight of root nodules, the level of the nitrogenase activity, plant biomassa and its nitrogen content. Electron-microscope studies and quantitative estimation of the Azospirillum cells have revealed the survival of bacteria on the root and especially on the root nodule surface during all the stages of ontogeny. The penetration of Azospirillum into the died cells of the cortex parenchyma and of the intercellular spaces was also shown. It is supposed that the production and release of IAA by Azospirillum are responsible for the stimulating effect of Azospirillum on legume-rhizobium symbiosis. The possible mechanisms of hormonal regulation caused by Azospirillum are discussed.

F-24 Screening of wheat genotypes for associative nitrogen fixation. M.I.Chumakov. Institute of Biochemistry and Physiology of Plants and Microorganisms, USSR Academy of Sciences. Pr.Entuziastov 13, Saratov 410015, USSR

The associative nitrogen fixation (acetylene reduction (AA)) activity (nis-trait) of 78 wheat cultivars and species of the genus Triticum L. with different genomes (A, AB, AG, ABD, AAG, AABG, AAGG) was investigated. AA-activity was found to depend on genome ploidy. The highest AA-activity was detected in hexaploid wheats (ABD, AAG genomes). Screening of 21 monosomic lines of wheat Saratovskaya 46 (ABD genome) for AA-activity showed the trait inheritance to have polygenic character. The most significant reduction of nis-activity was registered in 1A, 3A, 6D lines. Individual plants capable of supporting nitrogen fixation in soil bacteria population ("0 - plant") were noted within all wheat cultivars.

F-25 Dinitrogen fixation and apparent nitrogen transfer from legume to grass. G.H. Heichel* and K.I. Henjum. Plant Science Research Unit, USDA-Agricultural Research Service, and University of Minnesota, St. Paul, MN 55108-1013, USA.

The extent of N_2 fixation in legume-grass mixtures, and the importance of legume-grass N transfer, are poorly understood. A 4-yr field experiment was conducted to determine seasonal N_2 fixation and N transfer by stable isotope methods in swards of alfalfa, birdsfoot trefoil, white clover, and red clover with reed canarygrass. Alfalfa in mixture with grass fixed the most N_2 per season (82 to 254 kg N/ha) and white clover the least (1 to 20 kg N/ha). For all mixtures except trefoil-grass, N_2 fixation was greatest during Year 2. The proportion of legume N from fixation increased progressively from Year 1 through Year 4 and averaged over 93% across all legumes in Year 4. Legume-grass N transfer was greatest in Year 2 and averaged about 36% of grass N across all mixtures. On a land area basis, N transfer ranged from 29 to 53 kg N/ha and was greatest for the white clover-grass mixture. The decline in N transfer in Years 3 and 4 was associated with a loss in sward legume content, reduced dry matter accumulation in legume and grass, and large losses in root plus crown dry matter accumulation by grass in Year 4. The results indicate that forage legumes in mixture with grass can be virtually self-sufficient for N and can concurrently transfer appreciable N to the companion grass.

F-26 Nitrogen fixation and cycling of N in a pure stand of *Leucaena leucocephala*. C. van Kessel* and J.P. Roskoski. Dept. of Soil Science, University of Saskatchewan, Saskatoon, Canada, S7J 0W0, Canada, and National Science Foundation, Washington DC, USA.

A pure stand of *Leucaena leucocephala* (Lam) de Wit, a fast growing tropical tree, was established in 1986 on the island of Maui, Hawaii. The main objectives of the study were: (1) to estimate N_2-fixation activity by leucaena seedlings, and (2) to determine whether established leucaena continued to fix N. Leucaena seedlings, inoculated with rhizobia strain TAL 1145, were planted at a spacing of 1.5 by 1.5 m. The percentage of N derived from N_2 fixation was estimated using the $\delta^{15}N$ method. Deep and shallow rooted annual grasses in the vicinity of the test plot were used as reference plants. One year after planting the average $\delta^{15}N$ value of leaves and reference plants (5 species) was 3.9±0.3 and 7.3±0.4 ‰, respectively, which indicates that 43.0±4.9% of the N was derived from N_2 fixation. Two years after planting, the reference plant (14 species) $\delta^{15}N$ value decreased to 3.4±0.2 ‰. At this time, average leaf $\delta^{15}N$ value decreased slightly to 3.1±0.2 ‰, while branches had mean $\delta^{15}N$ values of 0.6±0.2 ‰. Two years after planting, total above ground N accumulation in leucaena was approximately 237±28 kg ha^{-1}, of which 80% was derived from N_2 fixation. Data indicate that N accumulated in two-year-old leucaena leaves was primarily derived from N mineralized from abscised plant components. N_2 fixation decreased with time as an equilibrium condition was approached between plant N uptake and net N mineralization of decomposing leaves and pods.

F-27

Selection of mungbean (Vigna radiata (L.) Wilczek) for high nitrogen fixation. R.Senaratne * and N.D.L.Liyanage. Dept. of Agronomy, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka.

Mungbean is a very important legume in Sri Lanka which is mainly grown in N-poor soils. Fertilization of this crop is erratic or almost non-existent. Consequently, it's productivity has remained very low (i.e. 0.75 m.t/ha as opposed to ca 2.25 m.t/ha under well-managed conditions). Hence, improvement of biological nitrogen fixation (BNF) shows promise in enhancing its productivity. Studies were therefore undertaken to screen mungbean germplasm for high BNF potential. Investigations involved over 100 genotypes which were tested under three management regimes i.e. without rhizobial inoculation, rhizobial inoculation with effective strain and fertilization at the rate of 60 kg N/ha. Observations were made on nodulation, plant growth, vigour and seed yield which were compared against a local check. A great variability in the above parameters was observed under each of the above regimes, and genotypes superior to the local check were identified. The ranking of genotypes in terms of yield depended upon the management regime, thus an interaction between the genotype and management regime was evident. Experimental details of the current and future investigations and results obtained will be presented.

F-28

N_2 fixation and agronomic evaluation of hypernodulation soybean mutants in the field. S. Wu* and J. E. Harper. Department of Agronomy and USDA/ARS, University of Illinois, Urbana, IL 61801, USA

Three hypernodulation mutants (NOD1-3, NOD2-4, NOD3-7) of soybean (*Glycine max*) were compared with Williams and a nonnodulation mutant (NN5) in a 2-year field study. Urea fertilizer treatments of 0 and 200 kg N/ha were imposed to test whether nodule formation and N_2ase activity of these hypernodulating soybean mutants would respond similarly to added N. Nodule activity was determined by acetylene reduction activity (ARA) of nodulated roots and relative abundance of ureides (RAU) in vacuum-extracted xylem exudate. The AR assay indicated that the NOD1-3 mutant was most tolerant of N in early stages of plant growth. Both the RAU technique (comparing NN5 with other lines) and the AR assay confirmed that nodulation of Williams was completely inhibited through the initial sampling date by N, while the three hypernodulation mutants showed detectable N_2 fixation activity. Based on RAU assays over the growing season, both NOD1-3 and NOD2-4 lines were more tolerant of N than was Williams. However, in the absence of N and at later stages of plant growth, N input from N_2 fixation by Williams was very similar to that of the three hypernodulation mutants. Yields from the hypernodulating mutants were 10-30% less than that of Williams. The results indicate that the hypernodulating mutants are currently inferior to Williams from an agronomic standpoint, although backcrossing is expected to improve their performance. The mutants may, however, be important in determining possible control points of the nodulation process.

F-29 Relationship between nodule development and nitrogenase activity during soybean pod setting. S. Bona*, R. Voltan and G. Mosca. Inst. of Agronomy, University of Padova, Via Gradenigo 6, 35131 Padova, Italy.

Nitrogenase activity (ARA) during "Hodgson 78" pod set was tested in growth chamber experiment in order to evaluate its relationships with number, weight and specific nitrogenase activity of nodules. Seeds were normally inoculated (INRA-G49) and plants were grown in a N-free nutrient solution. In R4 a water stress incidentally occurred. Total nitrogenase activity was greatly related to the development stage. It reached its maximum in R4 and it rapidly decreased in the last part of cycle. Nodule average dry weight and number of nodules did not change significantly during reproductive phases while the total nodule dry weight increased (from 0.75 to 1.25 g per plant). A multiple regression analysis of total nitrogenase activity per plant versus total nodule dry weight, nodule average dry weight, number of nodules and specific nitrogenase activity was performed. The results showed that the main factor affecting total nitrogenase activity was the specific activity ($P < 0.001$). The secondary factor appeared to be the total dry weight ($P < 0.001$). Number of nodules although significative in the multiple regression model ($P<0.05$) did not have an important role because of low value of partial regression coefficient ($\beta=0.02$). Only in the samples taken after the water stress the nodule average dry weight was significantly and negatively related to the total nitrogenase activity ($P<0.05$). This showed that, under water stress conditions, it is better for soybean to have nodules with low weight to guarantee a good nitrogen fixation. The number of nodules had only a relative importance during the reproductive stages but in R2 its effect was remarkable ($\beta=3.95$) and statistically significant ($P< 0.05$).

F-30 Variations in the measured nitrogenase activity of intact soybean nodules can be regulated by the undisturbed soybean plant. John Imsande, Dept. of Genetics, Iowa State Univ., Ames, IA 50011, USA.

Acetylene reduction activity (ARA) has been widely used to estimate comparative rates of nitrogen fixation. To establish the reliability of ARA measurements with soybean [Glycine max (L.) Merr.], nodulated plants were grown hydroponically and each plant was assayed for ARA twice weekly for 5 weeks. Plants were subsequently analyzed for total nitrogen by the microKjeldahl procedure. A comparison of the Kjeldahl and accumulated ARA values showed that, assuming an Electron Allocation Coefficient (EAC) of 0.75, ARA measurements for the 192 plants examined underestimated N_2 fixation by approximately 50%. Nitrogen fixation was underestimated by approximately 80% in plants with a very low ARA whereas N_2 fixation was underestimated by only approximately 20% in plants with high ARA. Nevertheless, for the 192 plants the correlation between accumulated ARA measurements and acquired nitrogen was approximately 0.70 with $P<0.0001$.

F-31 Use of herbicide resistant strains of diazotrophic cyanobacteria as biofertilizers in rice cultivation.

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The positive effect of diazotrophic rice cyanobacteria on the growth and yield of rice has led to their application as biofertilizers in rice cultivation. Since most of the cyanobacteria are sensitive to rice field herbicides, diazotrophic cyanobacteria resistant to these herbicides would be better suited for field application. In this respect several diazotrophic rice cyanobacteria, resistant to four herbicides, were selected by growth in the presence of field doses of herbicides and grown under culture conditions. These isolates represented mainly unicellular cyanobacteria (e.g.Gloeocapsa,Aphanothece,Chroococcus) and a few non-heterocystous cyanobacteria. No heterocystous forms could be selected for isolation. Results obtained on the N_2 fixation potential, photosynthetic O_2-evolution and diazotrophic growth showed that Gloeocapsa is capable of N_2 fixation in the presence of machete and basalin while Aphanothece could fix N_2 in the presence of diuron and propalin. Similarly the N_2-grown cultures of Gloeocapsa exhibited about 100-fold resistance to machete and basalin while cultures of Aphanothece showed 20-25 fold resistane to diuron and propalin, compared to that of Nostoc muscorum. GS-defective mutants of Gloeocapsa capable of liberating ammonia in presence of herbicides have been isolated.

F-32 Utilization of biofertilizers compensates for a yield reduction of vaccinated tomato plants.

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The use of satellite viral RNA [cucumber mosaic virus (CMV) associated RNA 5 (Carna 5)] as a biological control agent against severe CMV strains on tomato (Lycopersicon esculentum cv. UC82B) plants was found to cause a yield reduction (15-20%) from preincoculated or "vaccinated" plants.

To overcome this problem a greenhouse experiment was conducted using a mixture culture of nitrogen fixing bacteria of azospirillum (Azospirillum brasiliensis N040, Azospirillum brasiliensis SP7 and Azospirillum lipoferum MRB16) and one strain of blue-green algae [Anabaena oryzae (Fritsch)].

Treatments were control (unvaccinated) and vaccinated plants in combination with algae and/or azospirillum. Results showed that inoculation with azospirillum and/or algae gave significant differences in number and fresh weight of tomato fruits and plants' dry weight when compared to the control or vaccinated plants. Quality control studies were followed by polyacrylamide gel electrophoresis (PAGE) analysis of total nucleic acid extractions (TNA). The biofertilization was safe and did not affect the accumulation of viral satellite RNA required for plant protection.

F-33

Long-term effects on biological nitrogen fixing microorganisms by the herbicide 2,4-D. A. Mårtensson. Dept. of Soil Sciences, Div. of Plant Nutrition, Swedish University of Agricultural Sciences, P.O.Box 7014, S-750 07 Uppsala, Sweden.

EXPERIMENTAL BACKGROUND

In 1976 an accidental aerial application of 2,4-D was made in the forests of Potmäk, Sweden, (approx. 40 kg). The affected area discovered two months later was situated in an old spruce stand (30 x 60 m). All vegetation was dead and analysis of 2,4-D in soil was made immidiately. In 1979, 50% of the 2,4-D remained in the 0-5 cm top soil layer (100%=day of discovery). An adaptation of the soil microbes had taken place, the capacity for 2,4-D decomposition has improved. Soil respiration was reduced by 60-90% cf. control.

LONG-TERM EFFECTS ON BIOLOGICAL NITROGEN FIXING MICROORGANISMS

In 1989 the occurrence and activity of biological nitrogen fixing microorganisms at Potmäk was investigated. Endogenous nitrogen fixing bacteria and bluegreen algal populations were affected by 2,4-D, indicating that these organisms are suitable indicator organims when investigating environmental pollution, Table 1. No legume bacteria were found either in contaminated or control plots.

Table 1. Nitrogen fixing activities at Potmäk (10^{-14} mol ethylene/g soil and h)

	Distance from deposition (m)							
	0	10	17	36	45	72	92	control
Endogenous	0	0	0	53	57	38	132	623
Bluegreen algae	0	0	8885	8909	5277	5106	7673	10345

F-34

Effect of some pesticides on survival and growth of Rhizobium leguminosarum. E.M.[1] GEWAILY*, M.F. KHAN[2] and A.K. KHEDER[2], [1]- Agriculture Botany Department, College of Agriculture, Zagazig University, Egypt and [2]- Soil Science Department, College of Agriculture, Salahaddin University, Arbil, Iraq.

Insecticides (Kelthene, malathion, nogos and diptrex) and fungicides (rubigan, sulphur and benlate) have slight effect on rhizobia strains when applied at recommended doses. All cultures deteriorated when nogos and malathion were applied at 3500 and 3600 ppm, respectively. Rubigan was not highly toxic except at high concentration (96 ppm) for individual strains of rhizobia but the strain mixture could tolerate this effect for a certain extent. Sulphur and benlate had no detectable effect on different strains of rhizobia and their mixture. The sensitive levels on rhizobia differed with the strains as well as the pesticide used. In pots experiment nodules, dry weight and protein content of broad bean plants gave nearly the same trend as in vitro.

F-35 Effect of pesticides on growth and nitrogen fixation cyanobacteria Anabaena variabilis.

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Pesticides have been used these days in very large quantities because of their ability to improve agricultural production by controlling pests and diseases. They are usually aimed at target organisms but harmful effects may also be exerted on non-target organisms in soil. The growth and nitrogen fixation of cyanobacteria that are used as biofertilizers in rice cultivation is greatly affected in presence of pesticides. The effect of four pesticides (Carbaryl, BHC, Isoproturon and Gramaxone) in different concentrations has been studied in two strains of Anabaena variabilis. All the pesticides varied in their ability to affect the growth and nitrogen fixation of the two strains. In general, there was a marked increase in the initial lag phase and the exponential phase of growth got delayed by 6-7 days. Protein and chlorophyll content decreased with increase in pesticide concentration. Nitrogenase activity was comparable to control at lower concentration (0.5-1.0 ppm), however, it reached that level after a long lag period. The nitrogenase activity was greatly affected at higher concentrations.

F-36 Effect of cytozyme and bavistin on nitrogen fixation in *Vicia faba*.

B. N. Prasad, Central Department of Botany, Tribhuvan University, Kathmandu, Nepal.

Effect of different concentrations of cytozyme (bacterially derived high molecular protein extract which is basically a precursor of cytokinin) and bavistin (belonging to the benzimidazole group of fungicide) was studied on nodulation, nitrogen content and nitrogenase activity in *Vicia faba*. All doses of cytozyme showed stimulatory effect on fresh and dry weight of nodule and *in vivo* nitrogenase activity whereas the nitrogen content had a little or no effect. On the other hand, some concentrations of bavistin showed stimulatory effect whereas some concentrations had inhibitory effect on nodulation parameter and nitrogenase activity but on the nitrogen content it had a little or no effect.

F-37 Improvement in sustainability of arid and semi arid lands through nitrogen fixing trees. I.Hussain* and A.N. Mathur, Regional Bio-energy Centre, College of Tech. & Agril. Engineering (Rajasthan Agricultural University) Udaipur - 313 001 India.

Domestic fuel of rural areas primarily is consisted of firewood agricultural residues and animal wastes. Destruction of forests for formers and deprive of soil from later adversely affected sustainability of land. Trees particularly, nitrogen fixing, have systems of recycling nutrients through various plantations. Energy Plantation using NFT's were established at 11 locations (526 ha) in arid and semi arid regions representing saline-sodic, sandy, and degraded soils for biomass production and soil improvement. Among the species studied Prosopis juliflora, Acacia nilotica, Acacia tortilis, Prosopis cineraria, Pithecellobium dulce, Leucaena leucocephala and Acacia auriculiformis emerged promising for the purposes of energy plantation. Once the land scapes were devoide of trees have started producing fuelwood and grasses as an affect of energy plantation, thus sustainability was improved. The paper emphasizes use of NFT for energy plantation in arid and semi arid regions.

F-38 Evidence of Rhizobium cross-inoculation among fast-growing tree legumes used in agroforestry. D.S. Amara*. Department of Soil Science Njala University College PMB, Freetown Sierra Leone.

Pure Rhizobium isolates obtained from six tree and one annual legumes were used to inoculate seedlings of their host of isolation and other seedlings referred to here as their "heterologous host". The seedlings were sown in sterile sand medium and assessed for nodulation after 30 days. The results indicate that cross-inoculation occurs among the rhizobia. Three cross-inoculation groups have been identified with rhizobia from Albizia, Cajanus, Vigna nodulating the majority of the trees. This work has formed a basis for characterizing the tree legume rhizobia, and for preparing single or multistrain inoculants for use in soils where nodulation problems occur.

F-39

Seed molybdenum content in N_2-fixation of tropical Phaseolus vulgaris. Stephanie J. Brodrick*, Firoz Amijee and Ken Giller. Wye College, University of London, Wye, Ashford, Kent TN25 5AH UK

Molybdenum content of Phaseolus seed collected from farms and markets in East Africa ranged from being deficient (<0.08 µgMo/seed) to 20 times the amount required for growth and N_2-fixation (10 µgMo/seed). The relationship between parent seed content and growth, molybdenum content and N_2-fixation of offspring plants was investigated in the glasshouse with sufficient molybdenum or molybdenum deficiency.

Even when external sources of Mo were available to plants grown from large content seed (1.5-2.1 µg/Mo/seed) the seed reserve was the major source for growth and N_2-fixation until plants were 20 days old. Plants grown from this seed but not receiving molybdenum were sufficient for growth and N_2-fixation but produced seed of deficient molybdenum content. N_2-fixation and plant molybdenum content were reduced from 20 days after planting when seed of 0.24±0.152 µgMo/seed was grown without molybdenum. In all plants grown without molybdenum, irrespective of the seed content the nodules were extremely efficient scavengers gaining molybdenum at the expense of roots and shoots, and so preventing reduction in N_2-fixation.

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F-40

Accumulation of molybdenum in lupin seeds.N.P.L'vov*, K.L.Kalakoutsky,S.A.Bursakov. A.N.Bach Institute of Biochemistry Acad.Sci.USSR,Moscow,Leninsky pr.33

Legumes possess unique ability to molybdenum accumulation. It has been shown in our laboratory lupin plants,cultivated from Mo-enriched seeds possess higher nitrogen fixation- and nitrate reductase activityes as well as higher molybdocofactor contents than control plants. Biochemical mechanisms of molybdenum accumulation in legumes is unknown now. We have found that at least two proteins of lupin seeds extract are able to include of the biological analog of molydenum - tungsten (I85 - W) in both ways : in vivo and in vitro. We suppose that the proteins,mentioned above take part in the molybdenum accumulation in the lupin seeds, which are lack of any form of molybdenum cofactor (Mo-co).

SESSION G

Plant function
G-01 - G-59

G-01

Glycoprotein in intercellular spaces may control oxygen diffusion in soybean nodules. E. James* N.K. Brewin[1] J.A. Chudek F.R. Minchin[2] and J.I. Sprent. The University of Dundee, Dundee, DD1 4HN, Scotland. [1]AFRC Institute of Plant Science Research, John Innes Institute, Colney Lane, Norwich NR4 7HU England. [2]Welsh Plant Breeding Station, Aberystwyth, Dyfed SY23 3EB, Wales.

Many intercellular spaces in soybean nodules are occluded, rather then being filled with air. Specific glycoproteins, identified by monoclonal antibodies are a major component of the occluding material. To test whether the extent of air space occlusion is related to oxygen supply, plants were grown with nodules exposed continuously to 10, 21 or 40% oxygen for 28 days. Nodule sections were incubated with monoclonal antibodies and visualized using gold labelling (electron microscopy) enhanced with silver for light microscopy. The quantity of glycoprotein was directly related to the oxygen concentration under which nodules were grown.

Experiments with pure glycoprotein (gum arabic) suggest that its water retaining capacity, as visualized by proton NMR spectra may vary with ions present.

An hypothesis is put forward relating the extent of glycoprotein occlusions in air spaces to oxygen diffusion resistance.

G-02

α-glucosidase and malatdehydrogenase activity in the peribacteroid space of soybean root nodules.
A. Kinnback* and D. Werner. Philipps-University of Marburg, Dept. of Biology, 3550 Marburg, FR Germany

Bacteroids of *Bradyrhizobium japonicum* in root nodules from *Glycine max* are surrounded by a host derived membrane, the peribacteroidmembrane (PBM). The peribacteroid space (PBS), which is a lytic compartment between the outer membrane of the bacteroid and the PBM, was isolated by sucrose gradient centrifugation and the proteins were precipitated with ammoniumsulphate. The PBS contains a discrete set of proteins as revealed after one-dimensional SDS-PAGE. Marker enzyme activities proved no cross contamination with bacteroid cytoplasm and host cytoplasm. Several hydrolytic enzymes with a potentional role in plant defense could be detected in the PBS. Specific activity of α-glucosidase was 40-fold higher compared to host cytoplasm, very low activity could be detected in bacteroid cytoplasm and the enzyme was not present in root cytoplasm. Enzymes involved in the malat/aspartate shuttle such as malatdehydrogenase and glutamate-oxalacetat-transaminase were highly enhanced in the PBS. These results suggest that the PBS plays a major role in the regulation of the symbiosis.

G-03 **Plasmodesmata frequency selectively drops as infected soybean-nodule cells mature.**

Ernest Sutton[1], Evans Roth[1 2], and Gary Stacey[2 3]. [1]Department of Zoology, [2]Center for Legume Research, and [3]Department of Microbiology, University of Tennessee, Walters Life Sciences Building, Knoxville, TN 37996-0810.

Transfer of substances between nodule cells can be expected to take place through plasmodesmatal connections. We have determined their frequency in soybean nodules grown in a variety of conditions. In cells recently infected, plasmodesmata occur at similar frequencies between infected-infected, infected-uninfected, and uninfected-uninfected cells. However, as the infections mature, two changes are noticed. First, the plasmodesmata between two infected cells disappear, occurring at less than 1% of the number seen earlier. Second, those between two uninfected cells tend to cluster, so that patches of plasmodesmata appear to bind the two plasma membranes together locally, the cell wall there being thinner. These findings indicate the following. First, cells can eliminate plasmodesmata under certain conditions. Second, since adjacent infected cells lack plasmodesmata and therefore do not have confluent cytoplasms, they do not have plasmodesmata-related transfer; conversely, infected cells do have confluent cytoplasms with adjacent uninfected cells. Communicating groups of cells are indicated. Preliminary studies of mutant-induced nodules show that the plasmodesmata are maintained between infected cells, indicating that the signal to terminate plasmodesmata is probably not related to nitrogen fixation.

G-04 Arachis hypogaea Root Nodules: Ultrastructure reveals the presence of uninfected cells and enlarged peroxisomes.

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Arachis hypogaea root nodules develop by rhizobial infection without infection threads and they transport most of the fixed nitrogen as an amide 4-methylene-glutamine. We report here that these peanut root nodules are ultrastructurally unique from nodules produced by other legumes. Ultrastructure studies done on Arachis root nodules infected with rhizobia reveal rows of uninfected cells traversing across the multiple bacteroid zones. These uninfected cells are not interspersed among infected cells as commonly observed in other legume root nodules. The infected and uninfected cells also appear to be of similar size, a condition, markedly different from other amides or ureides transporting legumes. The uninfected cells reveal few but enlarged peroxisomes with an electron opaque central matrix and a clear "halo" in the outer part.

G-05

Effect of nitrate on peroxisome ultrastructure and catalase activity in nodules of Lupinus albus L. cv Multolupa. C. de Lorenzo. M.M. Lucas. A. Vivo and M.R. de Felipe*. U.E.I. Biología Vegetal. Instituto de Edafología y Biología Vegetal. C.S.I.C. Serrano 115 bis. 28006 Madrid, Spain.

This paper reports on an ultrastructural study of peroxisomes in infected nodule cells of Lupinus albus L. cv Multolupa plants grown with and without nitrate. Cytochemical localization of catalase and peroxidase was performed applying the diamino-benzidine (DAB) technique in these tissues.

The infected cells presented a mean of seven peroxisomes, the maximum being 16 in a few specimens. Peroxisomes shapes proved to be fairly round of egg-shaped, with maximum and minimum diameter means of 0.35 and 0.18 μm, respectively. They were preferably positioned on the cell periphery. The cytochemical data points out towards a strong catalase activity reaction in these microbodies in the form of intense osmiophilic staining.

Combined nitrogen addition (20 mol m^{-3}) to the growing plants exerted a negative effect on nitrogenase activity, which diminished by 31.6% after five days of treatment. Likewise, a reduction in catalase localization in the peroxisomes was observed, together with the existence of degenerative forms of the microbodies, whose ultrastructure appeared to be severely altered as well as that of bacteroids and peribacteroidal membres. By means of DAB, some darkening of the cytosol was observed, which could be due to an increase in peroxidase activity. Finally leghemoglobin localization was studied in nitrate-grown plants, confirming our previous observation of a decrease of this protein. It is suggested the involvement of these results in nodular tissue senescence following nitrate application.

G-06

The Fatty acid composition of the peribacteroid membrane and of the peribacteroid space material from Lupinus Luteus L root nodules.O.S.Genina, N.L.Radukina, K.A.Agibetov, G.Ya.Zhiznevskaya, A.G.Vereschagin, S.F.Ismailov. K.A.Timiriasev Inst. of Plant Physiology. USSR Acad. of Sciences, Moscow 127 276, Botanicheskaya35 USSR

The fatty acid composition of peribacteroid membrane (PBM) and of the peribacteroid space material (PBS) from root nodules formed on Lupinus luteus L after infection with an effective strain of Bradyrhizobium lupini 359a were analysed. The polymere 10 KDa with glucose as the main monomere was isolated from the PBS. The fatty acid C_{14}-C_{22} methyl esters from PBM and polimere were separated and quantified by gaschromatography. In the glucose-containing polymere of PBS the palmetoleic acid was found and the erucoic acid was absent, but in PBM, on the contrary, the content of erucoic acid consisted 12,26% of the total fatty acid content. The palmetoleic acid in PBM is absent. The content of palmitic acid in PBM was much higher (43,5% of the total fatty acids) than in polymere (26,32%) The absolute amount of fatty acids in PBM was 1,06 mg per mg of total protein. It's unknown yet whether the glucose-containing polymere is of bacterial or plant origin.

G-07

Immunolocalization of nitrogenase proteins in Frankia - Alnus incana symbiosis. [1] K. Huss-Danell* and [2] B. Bergman. [1] Dept of Plant Physiology, Univ. of Umeå, S-901 87 Umeå, [2] Dept of Plant Physiology, Univ. of Uppsala, S-751 21 Uppsala, Sweden.

Alnus incana (L.) Moench was inoculated with a local source of Frankia. Root nodule lobes showing acetylene reduction activity were examined for localization of nitrogenase. After a mild fixation in glutaraldehyde the nodule pieces were embedded in Epon or LRWhite. Antisera raised in rabbit against the Fe-protein (Rhodospirillum rubrum) and the MoFe-protein (Azotobacter vinelandii) were used. The immunoreactions were visualized in TEM with Au-conjugated (10 nm) anti-rabbit antibodies. Sections were taken from different regions in nodule lobes to include symbiotic vesicles of different ages, hyphae and spores of Frankia in the study. The distribution of the two nitrogenase proteins in the various forms of Frankia will be presented.

G-08

The "vesicle pore": a structure unique to stalked, septate symbiotic vesicles in actinorhizal plants. H. Berg* and L. Nichol, Department of Biology, Memphis State University, Memphis, TN, 38152, USA.

Frankia's nitrogen fixing symbiotic vesicles take on a variety of morphologies, depending on the host. In many actinorhizae, symbiotic vesicles are attached to the mycelium of the symbiont via a short hyphal segment termed the stalk. Critical analysis of thin sections shows that the stalk is covered by a remarkable number of cell wall surface lamellae, even more than are found surrounding the symbiotic vesicle per se. This results in compression of the stalk cytoplasm into a narrow channel, which we have termed the "vesicle pore". It is likely that this constriction mediates metabolite exchange between vesicle and hypha, perhaps making the symbiotic vesicle somewhat independent. Vesicle pores only occur in stalked, septate symbiotic vesicles (eg., in host genera Alnus, Colletia, Elaeagnus, and Hippophae) and Frankia vesicles in vitro. We have found no instances of in vitro Frankia lacking vesicle pores. The significance of the lack of a vesicle pore in stalked but nonseptate symbiotic vesicles is unknown, as is these symbiont's ability to form vesicle pores in vitro, since thin sections of these symbionts in culture are not available.

G-09

Cellular localization of xanthine dehydrogenase (XDH) in root nodules of cowpea [Vigna unguiculata (L.) Walp.]. E.H. Newcomb, D.B. Datta* and E.W. Triplett. Depts. of Botany and Agronomy, the University of Wisconsin-Madison, Madison, WI 53706, USA.

Cowpea, like soybean, is a ureide transporter. In ureide production via purine synthesis, early steps take place in the infected cells, and later steps in the uninfected. Localizing XDH will help identify the intermediate in ureide formation that moves from infected to uninfected cells. If XDH is entirely in the infected cells, this rules out a role for uninfected cells in purine synthesis for ureide production and points to uric acid as the intermediate. But if XDH is partly or wholly in uninfected cells, then the intermediate could be xanthine or a precursor, and purine synthesis in the uninfected cells remains a possibility. Recent attempts to localize root nodule XDH to cell type, and even to confirm its localization in the cytoplasm, are contradictory. Using polyclonal antibodies against cowpea XDH in an immunogold procedure, we obtained labeling in the ground cytoplasm of both cell types, with greater labeling in the uninfected cells. Controls with pre-immune serum showed no labeling. An artifactual origin for the labeling is ruled out by the virtual absence of label in the bacteroids, cell organelles and cell walls. We conclude that considerable purine synthesis for ureide production may be taking place in both cell types.

G-10

Specific interactions of rhizobial proteins (*trans*-acting factors) with distinct DNA sequence in the promoter region of *Sesbania rostrata* leghemoglobin (*lb*) genes. P. Welters*, G. Felix, J. Schell and F. J. de Bruijn. Max-Planck-Institut, 5000 Koln 30, F.R.G.

The establishment of nitrogen-fixing stem and root nodules on leguminous plants requires a fine-tuned interaction between rhizobia and their hosts. We are interested in the signal transduction pathways involved in nodule-specific expression of plant and bacterial genes involved in the symbiotic process. The plant genes specifically induced during nodule initiation and in mature N-fixing nodules have been called nodulin genes. The *lb* genes encoding oxygen transport proteins constitute primary representatives of late nodulin genes. We are studying the nodule specific induction of *lb* genes of the stem-nodulated tropical legume *Sesbania rostrata* (*Sr*) using chimaeric genes and transgenic plants. *Cis*-acting elements involved in nodule specific *Sr lb* gene expression have been identified. The interaction of *trans*-acting factors from different tissues with a conserved AT-rich motif repeated several fold in the *Sr lb* promoter region has been reported. Here we describe the characterization of a rhizobial protein, which specifically binds to a unique GC-rich element in the *Sr lb* 5′ upstream region. Footprinting analysis was used to delimit the binding site, which is conserved in the 5′ upstream regions of other nodulin genes. The protein is being purified using differential extraction, heat treatment of extracts and different chromatographic methods. Approaches to clone the corresponding *Azorhizobium caulinodans* gene and its mutagenesis will be described. The implications of these findings for endosymbiont mediated plant gene activation will be discussed.

G-11 Functional analysis of the *Sesbania rostrata* leghemoglobin *glb3* gene 5′ upstream region in transgenic *Lotus corniculatus* and *Nicotiana tabacum* plants.

L. Szabados*, P. Ratet, P. Welters, B. Grunenberg, J. Schell, and F. J. de Bruijn. Max-Planck-Institut, 5000 Koln 30, F.R.G.

Expression of the *Sesbania rostrata* leghemoglobin *glb3* gene was analyzed in transgenic *L. corniculatus* and *N. tabacum* plants, harboring chimeric *glb3-gus* (β-glucuronidase) gene fusions, in order to identify *cis*-acting elements involved in nodule-specific gene expression and general transcriptional control. Fragments of the *Sr glb3* 5′ upstream region with its own CAAT and TATA box regions or those of the Cauliflower Mosaic Virus 35S (CaMV35Sp) and nopaline synthase (*nos*) promoters, were fused to the *gus* reporter gene. These constructs were introduced into plants via *Agrobacterium* mediated transformation protocols and the expression of the reporter gene was measured both enzymatically and histochemically in different tissues. Distinct regions of the *Sr glb3* promoter required for nodule specific expression in *L. corniculatus* were thus identified. The *Sr glb3* promoter was found to be expressed to a low level in *N. tabacum*, primarily in the roots and phloem cells of the stem and petiole vascular system. In addition to nodule specific elements, upstream enhancer-like regions were identified. A comparison of the structure of the *Sr glb3* 5′ upstream region and those of other nodulin genes will be presented.

G-12 Stress-induced changes in nodule enzyme, leghaemoglobin and Lb-mRNA levels.

A.J. Gordon, Cell Biology Department, Welsh Plant Breeding Station, Aberystwyth, Wales, UK.

Changes in key nodule proteins following defoliation of white clover plants were assessed by measurements of enzyme activities and by use of antibodies to specific nodule proteins.

Defoliation caused major declines in protein and leghaemoglobin levels and in the activities of invertase, sucrose synthase, UDP glucose pyrophosphorylase, aspartate amino transferase, glutamine synthetase, PEP carboxylase and malate dehydrogenase. All increased again to control levels as new leaves emerged.

Antibodies to GS and Lb confirmed that the declines in these levels were due to disappearance of antigen. Similarly, antibodies to nitrogenase components I and II indicated that bacteroid proteins declined following defoliation and subsequently recovered in the same way as host plant encoded proteins.

In parallel studies with dark stressed soybeans an Lb cDNA probe was used to monitor Lb mRNA levels in total RNA extracted from nodules. Within 3 days of darkness Lb mRNA had disappeared whereas Lb protein had declined by only 20%. In plants returned to the light, Lb mRNA gradually reappeared as N_2 fixation began again.

G-13 NOVEL NODULIN cDNA CLONES ISOLATED FROM SOYBEAN ROOT NODULES BY A SUBTRACTIVE HYBRIDIZATION TECHNIQUE.

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Subtractive hybridization is a method to isolate cDNA clones of very rare messages. We applied this method to isolate nodule-specific (nodulin) genes from soybean root nodules of different developmental stages. ^{32}P-labelled cDNAs prepared from mRNAs of mature (21-day after *Bradyrhizobium* infection) and young (8-day) nodules were subtracted with large excess of mRNAs of uninfected roots and used to screen the cDNA libraries of the nodules. By this procedure, we isolated more than 15 different nodule-specific cDNA clones from the 21-day nodules. These include several unknown nodulin cDNAs as well as most of the nodulin clones previously reported. We also isolated several different cDNA clones including eNod-2 from 8-day nodules.

Expression of these novel nodulin genes during nodule development was investigated.

G-14 Nodulins in the course of effective and ineffective pea-Rhizobium symbiosis.

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The polyclonal antibodies raised against cytoplasmic proteins of pea nodules and purified on the CNBr Sepharose coupled with sterile root proteins were used to test nodulins. About 10 polypeptides (excluding leghaemoglobin) could be identified by Western blotting, most of them belong to the late nodulins as judged by time-course of accumulation during development. The gel filtration data showed that several of the immunoreactive polypeptides were subunits of high m.wt. proteins. Antibodied obtained were used to follow accumulation of the nodulins during symbiosis development of two Nod$^+$Fix$^-$ systems (one was based on noneffective Rh. strain, the other - on pea mutant line obtained by chemical mutagenesis). Majority of the nodulins were repressed in both systems, while quantaty of the specific protein 55kD increased significantly in nodules derived by noneffective Rhizobia.

In highly effective pea lines obtained by selection towards N_2fixation increase we described the minor changes in the nodulin pattern and coordinated enhancement of the several late nodulin genes expression.

G-15

ENOD2 localization in bacteria-free alfalfa nodules. C. van de Wiel[1], J.H. Norris[2]*, B. Scheres[1], R. Dickstein[3], T. Bisseling[1] and A.M. Hirsch[4]. [1]Dept. of Molecular Biology, Agricultural University, Wageningen, The Netherlands; [2]Dept. of Botany, Univ. of Rhode Island, Kingston, R.I. 02881 USA; [3]Dept. of Molecular Biology, Mass. General Hospital and Dept. of Genetics, Harvard Medical School, Boston, MA 02114 USA; and [4]Dept. of Biology, UCLA, Los Angeles, CA 90024 USA.

Alfalfa (Medicago sativa) plants form bacteria-free nodules in response to N-(1 naphthyl) phthalamic acid (NPA) or to 2,3,5-triiodobenzoic acid (TIBA), compounds which are auxin transport inhibitors (ATI). The nodules resemble bacteria-free nodules induced by Rhizobium meliloti exo mutants and Agrobacterium tumefaciens transconjugants carrying cloned R. meliloti symbiotic genes. All of these "enpty" nodules contain transcripts for the nodulins Nms 30 and MsENOD2; transcripts for late nodulins are not detected. In situ hybridization studies showed that ENOD2 transcripts were localized in the nodule parenchyma of normal root nodules. We found that the ENOD2 transcripts were expressed in nodule parenchyma in the bacteria-free nodules, but in some nodules the ENOD2 transcripts were localized to parenchymatous tissue at the base of the nodule and around the vascular bundles. We also checked nodulin gene expression in nodules induced by A. tumefaciens transconjugants, one harboring the R. meliloti nod/nif pSym and the other carrying both the pSym and the megaplasmid carrying exo genes.

G-16

Empty nodules of *Medicago sativa* transcribe ENOD2 but cells fail to show proper spatial organization. T. Allen and K. Dunn*. Dept. of Biology, Boston College, Chestnut Hill, MA 02167, USA

We have used *in situ* hybridization to examine the spatial organization of cells expressing early and late nodulin genes during nodule development in alfalfa. RNA transcripts from cDNA clones of ENOD2 and leghemoglobin (LB) were labeled with ^{35}S and used to probe paraffin sections of wildtype and mutant nodules. LB gene expression was restricted to the symbiotic zone and found only in those cortical cells infected with bacteroids. ENOD2 gene expression was found localized in the inner cortex of wildtype nodules, forming a boundary between the symbiotic zone and the outer layers of the nodule. Cells expressing ENOD2 surrounded the mature and senescent symbiotic zones, but were either absent from or dramatically reduced within the meristematic region. In contrast, ENOD2 gene expression in empty nodules elicited by Exo- *Rhizobium* was found concentrated in the meristematic region or in random cell clusters showing none of the internal organization characteristic of the wildtype nodules. In order to determine if the specific spatial grouping of cells expressing ENOD2 in wildtype nodules is a consequence of a more advanced development, we examined several Fix- nodules whose ultrastructure suggested fewer infected cells and a smaller mature zone of symbiosis. In each case, the cells expressing ENOD2 surrounded a smaller zone of symbiotic activity, suggesting that the spatial organization of these cells was connected functionally or structurally to the adjacent infected cells. These results suggest that transcriptional activation of the ENOD2 gene alone is not sufficient to organize those cells in their proper position within the functioning nodule. It is possible that either the invading infection thread or the release of bacteria into the cortical cells provides an additional signal necessary to direct the organization of the cells expressing ENOD2. To explore this possibility, we are currently examining wildtype nodules at various times post inoculation as well as additional Fix- nodules whose development is arrested prior to cortical cell infection.

G-17

The isolated Nms-25 (nodulin-25) promoter of alfalfa ensures nodule-specific expression in transform ed Medicago. G.B. Kiss, I. Meskiéne, J. Zabelina, and A. Gutkina. Institute of Genetics, Biological Research Center, Szege P. O. Box 521, H6701, Hungary

The promoter and the signal peptide region of the Nms-25 (nodulin-25) gene of alfalfa (1, 2) was placed in front of the structural gene of GUS (beta- glucuronidase) and cloned into a plant cloning vector pGA471 (3). Different tissue from the individual transformants of Medicago varia A2 (4) was tested for GUS activity as well as for the copy of the transformed DNA. The majority of the transformants showed nodule specific expression of GUS suggesting that the isolated promoter region is an active nodule specific promoter. Different level of GUS activity was detected in nodule, root, stem and leave tissue of some transformants which can be explained by "position effect" of the inserted DNA.

1. Kiss et al. Plant Mol. Biol. (1989 in press).
2. Végh et al. (1990, in preparation).
3. An et al. EMBO J. 4: 277-284 (1985).
4. Deák et al. Plant Cell Rep. 5: 97-100 (1986)

G-18

PHOSPHORYLATION OF NODULIN-26 BY A Ca DEPENDENT PROTEIN KINASE. **C. David Weaver[1]*, Gary Stacey[2], and Daniel M. Roberts[1], Dept. Biochem.[1] and Microbiol.[2] University of Tennessee, Knoxville, TN 37996.**

Nodulin-26 is a symbiosome membrane (SM) protein that is developmentally expressed in soybean nodules. In order to test whether nodulin-26 is a target of Ca-dependent phosphorylation we synthesized a peptide, CK-15, with the COOH-terminal 14 amino acids of nodulin-26. CK-15 is phosphorylated in a calcium-dependent, and calmodulin and phosphatidylserine/diacylglycerol-independent manner by a soybean nodule protein kinase. Anti-CK-15 antibodies recognize a protein from the SM with an apparent MW of 26,000 on SDS-PAGE. This protein is phosphorylated in a Ca-dependent manner. Consistent with the proposal that nodulin-26 is a nodule-specific protein, anti-CK-15 crossreactivity is observed with the SM fraction and not with samples from other tissues. Our data suggest that nodulin-26 may be a target of Ca-dependent regulation. **(Supported by NSF DMB 87-16224).**

G-19 Nodulin-26: Topology and function in root nodules.

G. H. Miao, C. P. Joshi and D. P. S. Verma*, Dept. of Molecular Genetics and Biotechnology Center, The Ohio State University, Columbus, Ohio 43210 U.S.A.

The amino acid sequence of nodulin-26 (N-26), a peribacteroid membrane protein from soybean root nodule was deduced from the analysis of a full-length complementary cDNA clone. The 271 amino acid protein has six transmembrane domains. This protein lacks a transient amino terminus signal sequence; instead, two internal putative signal sequences were identified. In order to determine the topology of N-26, a truncated sequence of the coding region corresponding to NH-terminal 91 amino acids (N-26T) was cloned behind a T_3 promoter in Bluescript plasmid and transcribed *in vitro*. The corresponding polypeptides were synthesized in rabbit reticulocyte cell-free system and their processing and insertion into dog pancreatic microsomal membrane were assayed by Con$^-$A binding, endoglycosidase H and trypsin digestion. These results suggest that N-26 is co-translationally inserted into the microsomal membranes. Since the N-26T is unable to insert into microsomes, the insertion of this nodulin in peribacteroid membrane may involve an internal signal sequence near the carboxyl terminus. The translocated N-26 is further modified by glycosylation as membrane-inserted N-26 binds to Con$^-$A.

Chemical cleavage mapping at cystein residues of trypsinized and untreated N-26 showed that both amino (N) and carboxyl (C) termini of the *in vitro* synthesized N-26 are sensitive to trypsin digestion, indicating that both N- and C-termini of this protein face outside of the peribacteroid membrane vesicle (i.e., facing the cytoplasm) and the glycosydic residues occur on the side of bacteroids. Based on our secondary structure model for N-26 on peribacteroid membrane and its comparison to the major intrinsic protein of bovine lens fiber membrane, it is very likely that N-26 may function as a channel or a transporter in soybean root nodules. Since the expression of this gene occurs prior to and independent of nitrogen fixation, N-26 may participate in translocating specific metabolites to support bacteroids.

G-20 Soybean nodule glutamine synthetase genes are regulated by ammonia in roots of transgenic *Lotus corniculatus*.

G.H. Miao[1], M.C. Marsolier[2], B. Hirel[2] and D.P.S. Verma*[1] [1]Department of Molecular Genetics and the Biotechnology Center, The Ohio State University, Columbus, Ohio 43210 U.S.A.; [2]Laboratoire du Metabolisme - INRA, Route de Saint-Cyr, 78000 Versailles Cedex, France

We had earlier demonstrated that glutamine synthetase (GS) activity in soybean root nodules may be regulated by the availability of ammonia, since the root tissue treated with ammonia for a short time transcribes high levels of GS mRNA. Isolation of a GS gene corresponding to this transcript (pGS15) followed by its insertion into *Lotus corniculatus* along with a reporter gene (GUS) showed that this GS gene is absolutely root-specific. Histochemical localization of GUS activity showed that this gene is primarily expressed in the cortical region of nodules and different parts of the root, the predominant activity being in the root meristem and vascular bundle. Treatment with ammonium nitrate induces this gene uniformly in the root tissue. However, the infected zone of root nodule shows a very low level of GUS activity. Another GS gene (pGS21) is also ammonia-inducible, but more specifcally expressed in the infected cells. Neither of these GS genes are inducible by ammonia when expressed in tobacco roots. These data suggest that the GS genes may be responding to the intracellular level of free ammonia and a root-specific factor present only in legumes. The low level of activity in the central region of the infected zone may be due to the lack of oxygen, since this tissue is highly anaerobic. This is demonstrated by the expression of alcohol dehydrogenase - GUS gene fusion experiments.

G-21

Affinity chromatography used to study nodule-specific proteins from soybean. K. Larsen and B. Jochimsen*, Department of Molecular Biology and Plant Physiology, University of Aarhus, DK-8000 Denmark.

Nodule-specific proteins have been identified using in vitro translation of nodule mRNA followed by immunoprecipitation with nodule-specific antibodies or by cDNA cloning followed by in vitro translation. Nodule-specific proteins (nodulins) are assumed to be involved in the development, structure and metabolism of the nodule, but the precise function of nodulins has only been established for a few: being enzymes (eg. glutamine synthetase, uricase, sucrose synthase) or oxygen carriers (leghemoglobin).
However such types of analyses will mainly reveal proteins occuring in relative high amounts. We chose affinity chromatography as a mean of concentrating nodulins occuring at around 0.1% of total protein.
Comparison of nodule extracts and root extracts eluting from Blue Sepharose by NAD additon and from Concanavalin A Sepharose by mannose addition revealed nodulins with subunit molecular weights of 40 kD and 30, 79, 94 kD, respectively.
Specific elution from more specialized materials might help to identify the function of such nodulins.
Two affinity materials: aminoimidazole carboxamide riboside and L-arginine coupled to Sepharose prooved to be excelllent in purifying uricase (nodulin-35). Uricase activity was retarded on both materials and repeated passage resulted in two polypeptide bands (33 and 31 kD) both representing uricase activity.

G-22

Extraction and cDNA cloning of mRNA from roots and nodules of European Black Alder (Alnus glutinosa L. Gaetrn.). P. Twigg* and B. C. Mullin. Department of Botany and The Center for Legume Research, The University of Tennessee, Knoxville, TN 37996-1100, USA.

Total RNA was extracted from Alnus glutinosa nodule and root tissue using a modification of the protocol developed by Dellaporta et al. (1983) for the extraction of DNA from plant leaves. This method was adopted after the efficacy of more conventional methods of RNA extraction (i.e., guanidine thio cyanate based on Chirgwin et al. [1979] and direct phenol extractions) were found to vary with the physiological status of the plant. Following RNA extraction, poly A mRNA was isolated from the total RNA of both root and nodule samples by oligo d(T) cellulose chromatography. These poly A fractions were used to produce nodule and root libraries in pTZ18R-B (Invitrogen). We are currently constructing nodule-specific cDNA libraries by employing the subtractive hybridization method of Duguid et al. (1988). The resulting nodule-specific clones will be further studied in order to determine their function and regulation during symbiosis.

G-23

The identification of proteins specific to symbiosis in the Alnus-Frankia actinorhiza. H. Belefant* and B. C. Mullin. Department of Botany and The Center for Legume Research, The University of Tennessee, Knoxville, TN 37996-1100, USA.

In the Alnus-Frankia symbiosis formation of root nodules capable of nitrogen fixation is dependent upon infection by a compatible Frankia strain. Very little is known about proteins uniquely involved in the symbiosis. In order to study such proteins, we have screened several protein extraction protocols for their suitability for use with Alnus, a woody plant high in phenolic compounds. On the basis of 1-D and 2-D polyacrylamide gels, we have identified several proteins that appear to be unique or present in altered amounts as a result of the symbiosis. These proteins are the target of further studies aimed at identifying factors responsible for their regulation.

G-24

Yellow lupin genes coding for the nodule-specific form of glutamine synthetase and (hydroxy)proline--rich protein, A.B.Legocki, L.Boroń, K.Szczygłowski. Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland

We present the structure and expression pattern of two nodule--specific genes from yellow lupin: 1. Glutamine synthetase genomic clone L1NGS (4.3 kb). A comparison of its structure with other legume GS genes reveals a similar exon-intron organization, We show the analysis of the promotor region, the transcription start site (its position is - 80 nucleotides upstream of the initiating AUG) and the location of regulatory motifs. 2. cDNA coding for (hydroxy)proline-rich protein (1551 nt) which is a homolog of early nodulin ENOD-2 containing the highly repetetive heptapeptide Pro-Pro-X1aa-X2aa-Lys-Pro-Pro. Northern blot analysis revealed that this lupin cDNA clone hybridizes with three mRNA species of 4000, 1800 and 1200 nt. A comparison of the lupin LENOD-cDNA clone with its homolog from soybean is presented.

G-25 **Studies on glutamine synthetase gene expression in nodules of French bean.** W.-J. Shen*, J. Freeman, M.S. Williamson and B.G. Forde, Dept. of Biochem. and Physiol., AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ, UK.

In French bean, almost all of the glutamine synthetase (GS) activity in nodules is specified by two of the five plant GS genes, *gln*-ß and *gln*-ɣ. The two genes are not coordinately regulated: *gln*-ß is expressed at high levels in roots and throughout nodule development, while *gln*-ɣ is expressed at low levels in roots but is strongly induced in nodules a few days before N fixation begins. Genomic and cDNA clones for both genes have been isolated so that their spatial and temporal patterns of expression can be analysed and the mechanisms regulating their expression investigated. In one approach, chimaeric gene fusions have been made between upstream GS gene sequences and the *gus* reporter gene and the constructs assayed in transgenic *Lotus corniculatus* plants. Fragments of 1.7 kb (*gln*-ß) or 2 kb (*gln*-ɣ) from the 5'-flanking regions of the genes were found to be able to direct the appropriate patterns of *gus* expression. We will report the results of a 5'-deletion analysis of the *gln*-ɣ promoter, the effects of *Rhizobium loti* mutants on expression of the *gln*-ɣ::*gus* gene, and a study of the role of ammonium in regulating *gln*-ß::*gus* expression

G-26 **Stabilization of glutamine synthetase protein by the toxin, tabtoxinine ß-lactam.** S.J. Temple*[1], T.J. Knight[2], P.J. Unkefer[2] and C. Sengupta-Gopalan[1]. Plant Genetic Engineering Labs/Dept. of Agron. & Hort., New Mexico State Univ., Las Cruces, NM 88003; National Labs, Los Alamos, NM 87545.

Psuedomonas syringae pv. 'tabaci', a commonly recognized leaf pathogen of tobacco can infest the rhizosphere of many plants, including oats. Normal oat plants do not survive this infestation as a consequence of the complete and irreversible inactivation of all their glutamine synthetase (GS) isoforms by tabtoxinine - ß-lactam (TBL), a toxin released by the pv 'tabaci'. We report here a significant increase in all GS protein isoforms (based on immunological determination) within a few hours of addition of the pathogen to the soil around the growing oat plants. Increase in total GS protein, however, is accompanied by a rapid drop in GS activity. Quantitative Northern blot hybridization of RNA from tissues of infested and uninfested plants, suggests that increase in GS protein as a result of pathogen infestation is not due to increased transcription of GS genes. Furthermore, results of short term in vivo labeling of tissues with radiolabeled aminoacids, also rules out the possibility of translational control as the mechanism for TBL mediated increase in GS protein. Taken together, the results suggest that the 10 fold increase in GS protein seen after 48h of treatment is due to the stabilizing effect of TBL on GS protein.

G-27 Characterization of a nodulin gene family in soybean. H. J. Richter*[1] and C. Sengupta-Gopalan[2]. Mol. Biol. Program. Dept. of Biol/[2]Dept. of Agron. & Hort., New Mexico State Univ., Las Cruces, NM 88003.

Root nodule development is accompanied by the expression of several sets of hosts genes encoding for nodule-specific proteins called nodulins. Among the several nodulin genes in soybean is a set of genes which are related to one another by the presence of extensive stretches of homology. The members of this gene family are transcribed at high levels in soybean nodules. Based on quantitative RNA hybridization studies, we find a much higher level of accumulated RNA for this gene family than for the leghemoglobin transcripts. Characterization of cDNA and genomic clones belonging to this family includes: i.Nucleotide sequencing and alignments of highly conserved domains; ii.Their hybridization pattern to nodule RNA; iii. Southern analysis of the soybean genomic DNA; iv. SDS PAGE profile of the translation products of nodule RNA, hybrid-selected with individual nodulin specific and gene family common DNA fragments. Based on these results we predict that this nodulin gene family is made up of at least seven functional genes. Two members of this gene family have been found to be linked to one another on the soybean genome. Our paper will also focus on the promoter analysis of members of this gene family.

G-28 Expression of genes encoding for phenylalanine ammonia lyase and chalcone synthetase and their possible role during nodule development in soybean. E. Estabrook* and C. Sengupta-Gopalan. Mol. Biol. Prog./Dept of Agron. & Hort., New Mexico State Univ., Las Cruces, NM 88003.

For the establishment of successful symbiosis between legumes and N_2-fixing bacteria Rhizobium/Bradyrhizobium species, the plant should be able to distinguish between a pathogen and a symbiont. This paper will focus on the expression of genes encoding for the enzymes, phenylalanine ammonia lyase (PAL) and chalcone synthetase (CHS), during nodule development in soybeans. PAL and CHS have a role, both as nodule initiaters and as defense molecules in host-pathogen interaction. Both genes show higher level of expression in developing nodules. To check if this increase in the level of PAL and CHS transcripts in the nodules, is due to the expression of a nodule-specific form of these genes, cDNA clones for CHS and PAL have been isolated from a soybean nodule cDNA library and their nucleotide sequence compared with published sequences of PAL and CHS genes expressed in ellicitor treated cells. Furthermore, the 2D gel profile of translation products of hybrid-selected root and nodule RNA have also been compared. To understand the specific role of these enzymes in nodule development, the pattern of expression of PAL and CHS genes have been analyzed in different ineffective associations between soybean and mutant strains of B. japonicum.

G-29 Malate transport and metabolism in infected cells of soybean nodules. Youzhong Li, Li-Jun Ou Yang, Rosanne G. Quinell, Michael K. Udvardi and David A. Day*. Botany Department, Australian National University, Canberra, 2601, Austraia.

Nitrogen fixation in soybeans is supported by recently-fixed carbon supplied to the nodule as sucrose. Recent evidence suggests that this sucrose is metabolized to organic acids, possibly malate, in the uninfected cortical cells, and then transported to infected cells. We have investigated malate uptake by isolated cells and the subcellular compartments within those cells.

Malate is readily accumulated by protoplasts isolated from the infected zone of soybean nodules. Uptake appears to be carrier-mediated and driven by mitochondrial oxidative phosphorylation. Sugars are not taken up at significant rates by protoplasts. Nodule mitochondria lack malic enzyme but possess a very active malate dehydrogenase and rapidly export oxaloacetate during malate oxidation. Malate oxidation is tightly coupled to phosphorylation. Malate can also be oxidized by cytosolic malate dehydrogenase. Malate transport across the peribacteroid and bacteroid membranes is rapid and occurs via distinct dicarboxylate transport systems. Malate is readily oxidized by bacteroids via NAD(P)-malic enzymes. Malate metabolism in infected cells thus has the ability to provide the carbon skeletons and energy required for both nitrogen fixation in the bacteroid and ammonia assimilation in the plant cytoplasm.

G-30 Protein synthesis of *Bradyrhizobium japonicum* bacteroids from soybean root nodules. Dale B. Karr* and David W. Emerich, Dept. of Biochemistry, University of Missouri, Columbia, MO 65211

In *Rhizobium*-legume symbiosis, protein synthesis has been described for the fast-growing species, but not for the slow-growing *Bradyrhizobium japonicum*. In bacteroids of the fast-growing *Rhizobium*, protein synthesis declined with nodule age, was active, predominately, in those isolated from the apical nodule region, and synthesis of a number of proteins, including nitrogenase components appeared continuous but not coordinated. In this report, the characterization of the protein-synthesizing apparatus of the *Bradyrhizobium japonicum* bacteroids from soybean root nodules is presented. In *B. japonicum* a small decline in the level of protein synthesis was observed during nodule development. Levels were about 10% of that in cultured cells. The only observable differences in the pattern of ^{35}S labeled proteins, as a function of nodule age, were a relative increase in intensity of label in the 60 kd region and decrease at about 35 kd, otherwise the patterns were similar. The patterns of phosphorylated proteins, labeled *in vitro*, were similar over the period studied but the intensity of label incorporated in bacteroid proteins from older nodules was less than that in bacteroids from younger nodules. Levels of protein synthesis and phosphorylation in response to environmental stimuli are being studied.

G-31

Purification and characterization of allantoinase from soybean root nodules. Mary Alice Webb* and Judy Lindell. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47906, USA

Allantoinase catalyzes the final step in ureide biogenesis in soybean root nodules. It has been proposed that allantoinase is localized in the tubular endoplasmic reticulum (tER) found in the uninfected cells of ureide-producing root nodules. However, ultrastructural studies have shown that the pattern of tER development and proliferation do not match reported patterns of allantoinase activity in these nodules (Webb and Newcomb, 1987, Planta 172:162). The goal of recent studies has been to purify allantoinase and prepare antibodies against the enzyme for use in immunolocalization studies. Improvements have been made in a previously-reported protocol (Webb et al., 1987, Plant Phys. 83:21) for allantoinase purification from soybean root nodules to give substantially better yield. A crude extract of soybean nodules was centrifuged at 30,000 x g, and the supernatant collected and subjected to heat treatment at 72°C for 10 min. After centrifugation to remove heat-precipitated protein, the supernatant was fractionated with ammonium sulphate, and the fraction precipitating between 45 and 75% ammonium sulphate was collected and redissolved in buffer. Two volumes of cold chloroform:ethanol, 1:2, were added to precipitate leghemoglobin, which was the major contaminant at this stage. Cold ethanol was added to the extract and the fraction precipitating between 50 and 70% ethanol was saved for further purification by column chromatography. This procedure resulted in 47-fold purification with 37% yield after the ethanol fractionation, compared to 34-fold purification with 11% yield at a comparable stage of the procedure previously developed. Partially purified protein has a broad pH optimum with a peak at pH 7.0 and a K_M of 50 mM.

G-32

Factors influencing the sap ureide composition in soybean inoculated with B. japonicum. N.G. Rumjanek*, R.C. Peixoto and M. C.P. Neves. EMBRAPA, CNPBS Seropédica km 47 RJ 23851, Brazil.

A positive correlation has been shown between the hydrogenase present in the nodule and a high sap N-ureide %. Recently, we have observed a further increase in this parameter when the nodule environment was enriched with H_2. Nevertheless, some authors are not being able to detect this correlation which may be a result of others factors. Soybean was inoculated with B. janponicum HUP^+ and HUP^- and sap was collected as expontaneous bleeding or vacuum extracted. The ureide transport rate of expontaneously bleeding sap showed an increase of 16,2% for the soybean inoculated with the HUP^+ strain compared to the HUP^- one. Vacuum extracted sap showed an even more significant difference, the HUP^+ strain was able to induce an average increase of 28,5%. In another experiment, sap was collected during 1 hour in several aliquots. The differences in the ureide concentration between HUP^+ and HUP^- strains were influenced by the interval of time at which the sap aliquots were collected. Data from different cultivars will also be discussed.

G-33

Presence of insoluble nitrate reductase in legume root nodules. A. Kaur*, C. Arrese-Igor, A.J. Gordon and F.R. Minchin. Department of Biochemistry, University of Horticulture and Forestry, P.O.Nauni 173230,Solan, India.

In vitro assays of nitrate reductase activity (NRA) in nodules of soybean and mung bean have detected constitutive NADH dependent activity, with about 50% of this activity being in the 8,000g pellet. The total (pellet + cytosol) nodule activity increased during an 8 day application of nitrate. A constitutive methyl viologen dependent NRA, of particulate nature, was present in bacteroids. However, no NADH dependent activity was associated with bacteroids. No constitutive NRA could be detected in homogenates of roots and, following nitrate application for 4 days, only 5% of activity was detected in the 8,000g pellet. This could be explained by the presence of unbroken cells in the homogenate.

The insoluble NRA of nodules was not removed by repeated washing of the pellet or disruption of nodule cells using a ground glass tissue homogenizer. Following this treatment light microscopy observations suggested that 90-95% of all cells had been broken. It is yet to be determined as to whether this previously unreported insoluble NRA represents a membrane bound enzyme.

G-34

Effect of nitrate on malate dehydrogenase and aspartate amino-transferase activity and on several metabolites in lupin root nodules (Lupinus albus L. cv Multolupa).M.P. Golvano*and P. Lang. U.E.I. Biología Vegetal. Ins. Edafología y Biología Vegetal. Serrano 115 bis. 28006 Madrid, Spain.

In this work we describe the methods followed to isolate the bacteroids and mitochondria from cytosol in lupin nodules and we present data on the effect of nitrate on the specific activity of MDH and AST in the different cellular fractions. The change in the level of some metabolites have also been studied. The bacteroids showed higher MDH activity than cytosol, 3-fold in the direction of oxaloacetate reduction and 5-fold in the direction of malate oxidation. However, reduction of oxaloacetate was the highest activity in both cytosol and bacteroids. The enzyme AST operates in the cytosol mainly in the direction of aspartate utilization and favouring aspartate formation in the bacteroids. In the mitochondria, the predominant reactions were reduction of oxaloacetate by MDH and aspartate formation by AST. Supply of nitrate (20 mM) in short-terms studies produced a trasient increase of the bacteroids MDH and AST specific activity, but after there declined. After 2 d of treatment an increase of 50% in the direction of oxaloacetate reduction and 74% in the direction of malate oxidation by MDH was observed. AST activity increased by 76% in the direction of aspartate utilization. Nitrate supply increased malate in the nodule cytosol while oxaloacetate and cetoglutarate accumulated in bacteroids. Aminoacids mainly aspartate decreased in bacteroids and cytosol. If nitrate restrict O_2 diffusion to bacteroids, the transient increases in MDH and AST activities might be a mechanism to oxidize the NADH which can not be oxidized by electron transport chain.

G-35 **Biochemical properties of monodehydroascorbate reductase from soybean root nodules. David A. Dalton*, Lorene Langeberg, and Michael Robbins. Biol. Dept., Reed College, Portland, OR 97202, USA**

Legume root nodules contain the enzymes of the ascorbate-glutathione pathway to scavenge potentially destructive H_2O_2. This cycle involves the regeneration of reduced ascorbate in a reaction catalyzed by monodehydroascorbate reductase. This enzyme was purified 440-fold to near homogeneity by use of ion exchange, gel filtration, and affinity (Blue Sepharose) chromatography. It contained FAD and had a molecular weight of 43,000. The pH optimum was 8.5. The K_m was 5.6 μM for NADH and 150 μM for NADPH. The purified enzyme had no detectable dehydroascorbate reductase activity. The purified enzyme was not stable under various freezing regimes, but could be stored in 50% glycerol at -20°C without loss of activity.

G-36 Glyceraldehyde-3-P dehydrogenase from the plant cytosol of soybean nodules. L. Copeland* and A. Zammit. Dept of Agricultural Chemistry, Univ. of Sydney, NSW 2006, Australia

NAD-dependent glyceraldehyde-3-P dehydrogenase catalyzes a key step in the glycolytic sequence. The enzyme has been studied extensively from animal and microbial sources but only to a very limited extent from plants. This study, which is part of a continuing investigation on the metabolism of carbohydrates in association with symbiotic nitrogen fixation in legumes, is concerned with the NAD-dependent glyceraldehyde-3-P dehydrogenase from the plant cytosol of soybean (*Glycine max* L. Merr. cv Williams) nodules. The enzyme was purified to homogeneity and shown to have regulatory properties that could be involved in controlling the flux through the glycolytic pathway. The enzyme was very labile, with loss of activity being accompanied by changes in the aggregation state of the protein. The substrates, NAD and Pi, were required to prevent the decay of activity. A polyclonal antiserum, raised in rabbits against the soybean nodule glyceraldehyde-3-P dehydrogenase, and shown to be monospecific by Western blot analysis, cross reacted with the enzyme in extracts from soybean roots, leaves and stems, as well as from nodules of other legume species.

G-37

Soybean nodule phospho*enol*pyruvate carboxylase: Partial purification and regulatory properties. K.A. Schuller*, D.H. Turpin and W.C. Plaxton. Biology Dept., Queen's University, Kingston, Ontario, Canada K7L 3N6.

One form of phospho*enol*pyruvate carboxylase (PEPC) was detected in soybean (*Glycine max* cv. Maple Arrow) nodules. The partially purified enzyme exhibited a broad pH optimum of between pH 7.5 and pH 9.5. The response of PEPC activity to PEP concentration was hyperbolic and the K_m(PEP) was 0.08 mM at pH 8. The effects of various metabolites at this PEP concentration were determined at pH 7.5 and pH 8. Malate, aspartate and glutamate were strong inhibitors at pH 7.5 but had little effect at pH 8. Similarly, glucose-6-phosphate and fructose-6-phosphate were notable activators at pH 7.5 but had little effect at pH 8. In contrast, pyruvate, 3-phosphoglycerate and citrate were equally inhibitory at both pH values. The effects of malate, aspartate and glutamate were not additive suggesting that they all bind at the same inhibitory site. Succinate, 2-oxoglutarate and a range of amino acids including glutamine and asparargine had no significant effect on PEPC activity at either pH value. The results are discussed in terms of metabolite concentrations in soybean nodules and the role of PEPC in this tissue.

G-38

Photosynthate partitioning and regulation of soybean (Glycine max L.,Merr) nodule development. J.I.Hacin, B.B.Bohlool*, and P.W.Singleton, NifTAL Project, University of Hawaii, Paia, HI 96779.

Photosynthate (PS) partitioning to developing soybean roots and nodules was evaluated in a split-root growth system. Two inoculation treatments with B.japonicum (USDA 110) were imposed: 1) E/U -one side inoculated early, the other remained uninoculated; 2) E/D - one side inoculated early, the other side inoculated with a four day delay. Plants were labeled with $^{14}CO_2$ and harvested at 24 h intervals, from inoculation until the onset of N_2 - fixation (acetylene reduction). Roots and developing nodules were separated into eight categories, based on structure and developmental stage. Selective partitioning of PS to these categories was characterized by autoradiography and scintillation counting. Sink intensity, rate of nodule development, and the number of infections (sum of observations of four developmental stages from cortical cell divisions to functional nodules) on early and delayed sides suggest, that PS partitioning might play a major role in regulation of nodule development.

G-39

The accumulation of lectin in peanut nodules in relation to the symbiotic effectiveness of Rhizobium strains.

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In previous studies a lectin antigenically similar to peanut seed lectin was detected in peanut nodules. In order to determine whether the amount of lectin accumulated in nodules is affected by the effectiveness of rhizobia, three peanut (Arachis hypogaea) varieties (Virginia, Spanish and Valencia) were inoculated with highly effective, mediocre and non-effective Rhizobium strains. Enhanced levels of lectin/gram nodule tissue as measured by ELISA (enzyme-linked immunosorbent assay) have been found in plants inoculated with highly effective rhizobia. Significantly more lectin accumulated in the nodules of two Virginia cultivars grown without mineral N than in nodules of N-fertilized plants. Our results on the lectin levels in nodules induced by Tn5 (Nod^+,Fix^-) Rhizobium mutants will be presented.

G-40

MANNOSE- AND GALACTOSE-BINDING LECTINS FROM NODULES AND OTHER TISSUES OF PEANUT.

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Mannose-binding lectin (ML) was purified from all tissues of 6-week-old nodulated peanut plants but galactose-binding lectin (GL) could only be purified from nodules. Nodule ML and GL each had similar sugar specificities to corresponding cotyledon ML and seed GL but differed in sensitivity to haemagglutination inhibition by sugars and glycoproteins. Nodule and leaf ML were identical in their reactions with sugars and glycoproteins. Neither nodule lectin reacted with cells or bacteroids of peanut rhizobia. Nodule ML was antigenically partially identical to cotyledon ML but identical to ML from other tissues. Nodule ML isolectins resembled those of ML from cotyledons and other tissues. The amino acid composition of nodule and cotyledon ML was similar but their carbohydrate content and composition differed. The glycoprotein nature of nodule GL was confirmed; seed GL is non-glycosylated, Nodule GL had a major isolectin that differed from the six isolectins of seed GL. Despite similar amino acid composition only four of the first 10-terminal amino acids of nodule GL corresponded in position to those of seed GL. Nodule and seed GL appear to be products of different genes.

G-41

Functional expression of pea lectin on root hair tips of transgenic white clover hairy roots

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Introduction of a gene encoding pea lectin, psl, into white clover hairy roots extended the range of nodulation of clover roots to Rhizobium leguminosarum biovar viciae (1).

By using a light-microscopic localization method based on specific binding of pea lectin, Psl, to the carbohydrate moiety of rat IgE, we were able to demonstrate the presence of Psl on the surface of root hair tips of transgenic clover roots, similar to the situation with pea roots. These results show that Psl on pea roots and transgenic clover roots is expressed in a biologically active form.

1. Díaz, C.L. et al. (1989) Nature 338, 579-581

G-42

Lectins in pea genotypes with different nodulation ability. M. Gräfin Schmettow*, H.P. Müller, Dept. of Biochemical Genetics, University of Bonn, D-5300 Bonn, Federal Republic of Germany.

Lectins seem to fulfill a vital role in specific recognition between roothairs of Leguminosae and nitrogen-fixing Rhizobiae. Mutants and recombinants of Pisum sativum, var. DGV, were screened for nodulation ability in field trial and in-vitro-assay. Mutant 3137 with very low nodulation has at least 10fold lower agglutination activity in seed extracts. Electrophoretic investigations (SDS-PAGE, IPG, 2D-PAGE) demonstrate that one of the isolectins is missing and one subunit changed in amount. Our results support the theory about the important role of lectins in initiation of nodulation leading to symbiotic nitrogen-fixation.

G-43

Inhibition of nodulation on one side of a split-root-system of peas resulting from prior NO_3^--supply to the other side. W. Schmidt*, P. Martin. Institut für Pflanzenernährung, Universität Hohenheim, Postfach 70 05 62, D-7000 Stuttgart 70, F.R.G.

Two experiments have been carried out to investigate whether the inhibitory effect of NO_3^- on nodule initiation and development in legumes depends on an interaction between NO_3^- and an autoregulation signal in plants. Pea plants were grown using a split-root system (flow-through culture) in which the nutrient solution contained a small amount of NO_3^- (0.5 mM). One side of the roots was supplied with 200 ml of a 25 mM NO_3^--solution daily for 2 days (Expt.I) or daily for 4 days (Expt.II) before inoculation of the other side which was carried out when the plants were 18 days old. Roots of the $+NO_3^-$-side of plants were cut off just prior to inoculation. A significant reduction of nodule number/root occurred in Expt. II but not in Expt. I. At the time of inoculation plants of both treatments had higher contents of total N and NO_3^- in shoot and roots (which had not received NO_3^-), higher concentrations of amino-N in the shoot and lower contents of water-soluble carbohydrates in shoot and roots than the control plants. There was only a slight difference in these parameters, however, between plants of Expt. I and II. Results suggest that NO_3^--supply for several days prior to inoculation activates an internal regulating system which controls nodulation of pea.

G-44

Early Nodulation Signals in Wild-Type and Symbiotic Mutants of Soybean
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Release of low molecular weight flavonoid signal molecules from soybean plants stimulates invasion and symbiotic nitrogen-fixation by the *Bradyrhizobium japonicum* soil bacterium. The "common" nodulation genes of *Bradyrhizobium, nod(Y)ABC* are essential for nodule initiation and are induced by plant flavonoid signals.

We used expression of the *nodYABC* operon to monitor changes in early nodulation signal levels of wild-type, supernodulating (autoregulatory) and non-nodulating mutants of soybean. This was done to determine whether variations plant signal levels correlate with nodulation phenotype.

The validity of the *nod* gene induction bioassay was confirmed using HPLC analysis which showed that peak size of the flavonoid inducer daidzein in fractionated extracts correlated with *nod* gene inducibility.

Analyses of extracts from seeds, cotyledons, roots, stems and leaves showed levels of *nod* gene induction characteristic for each tissue type; however, no significant variation was observed between each plant genotype tested. Root tip extracts from inoculated and uninoculated plants and exudates from each plant genotype also showed no significant differences in *nod* gene inducibility.

Our results indicate that autoregulatory and non-nodulating soybean mutants do not have significantly different levels of bacterial *nod* gene signalling molecules compared to wild-type plants. It appears unlikely that alterations in levels of plant signals are responsible for the mutant symbiotic plant phenotypes examined.

G-45

Distribution of *Bradyrhizobium*-induced cell divisions in soybean.

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Soybean nodules can only be initiated in a susceptible zone between emerging root hairs (EH) and root tip (RT), defining a spatial window that moves acropetally with root development. Cell divisions lead to clusters of cells capable of meristem formation and differentiation into nodular tissues. Nodulation is also autoregulated–early nodules inhibit later nodule formation. This control begins within 3 days post-inoculation and is maximal after 7 days. Nodule number reaches a plateau at 13 days. Since nodule maturation takes about 6 days from infection, the plateau may represent autoregulation closing a developmental window which all but committed pre-nodule cell divisions have missed. Induced cell divisions from main roots were scored in a spatial window 13 days post-inoculation. Cell divisions were observed at stages III or IV (Calvert *et al.* 1984) with few, if any, other stages prior to mature nodules (stages X-XX) present. Nodule number represented about 0.8% of the total cell division events observed. Events were restricted mainly to the two relative distance units (=EH-RT distance at inoculation) below the initial susceptible zone, although a significant number of events also occurred above this zone. Since most cell division stages in fully autoregulated roots were at stage III-IV and the number of cell division events in wild-type and an autoregulation-minus mutant are similar (Mathews *et al.* 1989), it appears that autoregulation blocks nodule development and not nodule initiation. The developmental blockage of nodule emergence which results in the accumulation of stages III and IV appears to coincide with meristem formation.

Calvert *et al.* (1984) Can J. Bot. **62**:2375-2384, Mathews *et al.* (1989) Protoplasma **150**:40-47.

G-46

Ultrastructural development of spontaneous nodules in alfalfa.

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The ontogeny of spontaneous nodules in alfalfa (*Medicago sativa* L. cv Vernal) was studied in serial longitudinal plastic sections of roots at different stages of growth, with light and electron microscopy. These nodules in uninoculated roots were white multilobed structures, histologically composed of nodule meristems, cortex, endodermis and a central zone with vascular strands. Early development of nodulation was initiated by anticlinal followed by periclinal divisions of dedifferentiated cells in the inner cortex of the root. These cells formed the nodular meristem from which the nodule developed. Each cell division in the meristem formed one large and one small cell which persisted in the central zone. The large cells were characterized by a large central vacuole and cytoplasm packed with starch, while starch was present only occasionally in the small cells. Instead of starch, numerous lipid bodies occupied the small cells. Cells in the meristem and central zone contained fragmented rough endoplasmic reticulum, numerous free ribosomes, and few organelles. There were no infection tubes or bacteria in spontaneous nodules at any stage of development. The large cells became infected with bacteria in normal nodules (inoculated plant) whereas they accumulated starch in spontaneous nodules. Alfalfa plants apparently have an endogenous mechanism for inducing early cell divisions which initiate nodular meristems and ultimately develop bacteria-free nodules.

G-47

Spontaneous nodules induce autoregulation of nodule formation in alfalfa.
G. Caetano-Anollés, P. A. Joshi and P. M. Gresshoff.* Plant Molecular Genetics, Institute of Agriculture and Center of Legume Research, The University of Tennessee, Knoxville, TN 37901-1071, USA

A small subpopulation of alfalfa (*Medicago sativa* (L.) cv. Vernal) plants grown without fixed nitrogen can develop nodules in the absence of *Rhizobium* (as described by Truchet *et al.*, Mol. Gen. Genet. 219:65-68, 1989). These spontaneous nodules appear mainly on the primary roots and are initiated between 4 and 6 days after imbibition of the seed. Cytological studies showed that these nodules were organized root-derived structures with no inter- or intracellular bacteria. Few if any viable bacteria were recovered from the nodules after surface sterilization. When the contents of the nodules were used to inoculate alfalfa roots no nodulation was observed. Nodules appeared as early as 15 days after germination with a rate of emergence comparable to that of normal nodules. Once these nodules were formed, we examined their ability to control nodule initiation in other parts of the root system. The formation of spontaneous nodules on the primary root suppressed nodulation in lateral roots after inoculation with *R. meliloti* strain RCR2011. Subsequent excision of these spontaneous nodules allowed for a further increase in lateral root nodulation. Our results indicate that the presence of *Rhizobium* is not required for the elicitation of feedback regulation of nodule formation in alfalfa and suggest that induction of cortical cell division in the roots is the crucial event in nodulation control

G-48

Alternative mechanisms control nodulation in legumes
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Nodulation is controlled by an autoregulatory response that suppresses nodule emergence once first nodules are formed. In a set of cytological and physiological studies of nodule initiation and development in soybean (*Glycine max* (L.) cv. Bragg) and alfalfa (*Medicago sativa* (L.) cv. Vernal) we found distinct differences in the way feedback suppression of nodulation was exerted. Soybean plants grown in pouches developed nodules on both primary and secondary roots. In contrast, nodulation in alfalfa was restricted to the primary root. Excision of primary nodules in soybean caused nodules to reappear in the same regions from where nodules were originally excised. In alfalfa, nodule removal from the primary root resulted in nodulation occurring only in lateral roots in regions that were susceptible at the time of excision. Anatomical studies showed that almost all infections associated with cortical cell division in alfalfa developed into functional nodules. In soybean, only a small fraction of cell division foci mature to form nodules, while nodule initiation appears constant throughout root development. Our results suggest that at least two divergent mechanisms control nodulation in legumes. One exerts feedback suppression of nodule formation through the arrest, and perhaps abortion, of nodule development, the other controls nodule initiation presumably during pre-infection.

G-49 Developmental and Biochemical Regulation of 'Constitutive' Nitrate Reductase Activity in Leaves of Nodulating Soybean.

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The developmental profile of total 'constitutive' nitrate reductase activity (cNRA) in leaves of soybean (*Glycine max* (L.) cv. Bragg) plants was investigated. The youngest leaves had the higher cNRA which dropped off as a newer leaf developed. Each leaf had its distinct active period of cNRA. This pattern differed between urea-grown and symbiotically-grown plants (inoculated with *Bradyrhizobium japonicum* strain USDA110), where the latter had no detectable cNRA in older leaves, while urea-grown plants maintained considerable activity in such older leaves. Changes of leaf cNRA related to the extent of nodulation. cNRA in leaves was controlled by functional (i.e. nitrogen fixing) nodules. When symbiotically-grown plants had their nodules excised, cNRA reappeared in older leaves within one day of removal. Allantoic acid was implicated as a possible signal molecule from the nodules to leaves. Allantoic acid (at 400 μM) completely inhibited *in vitro* c_1NRA. Inhibition was by a competitive process. Kinetics showed a constant Vmax of 105 nmol NO_2 mg^{-1} protein h^{-1} and a Km for nitrate of 15 mM, which increased to 60 mM in the presence of 200 μM allantoic acid. Allantoic acid did not inhibit inducible NRA. These findings raise the possibility that c_1NRA possesses another biological function, perhaps related to ureide metabolism. The selective inhibition of c_1NRA by allantoic acid may provide a method to evaluate charges in the distribution of nitrate reductase in different soybean genotypes or plants grown under different nitrogen regimes.

G-50 Nitrate inhibits nodulation of supernodulating soybean mutants.

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Several nitrate tolterant symbiosis (nts) mutants of soybean have been isolated which continue to nodulate with continuous additions of NO_3 in sand culture. These mutants also produce many more nodules (supernodulation) than the wild type. However, the response of these mutants to NO_3 when grown in soil has not been investigated, and there is little quantitative information available concerning the amount of N_2 fixed by the nts mutants. Plants were grown to physiological maturity in 3 kg soil. Nitrate levels of 7.5, 150, 300, or 450 mg NO_3-N/pot (equivalent to 5, 100, 200, or 300 kg N/ha) were added at planting. The ^{15}N isotope dilution technique was used to estimate N_2 fixed. Increasing the NO_3-N level from 7.5 to 150 mg/pot did not significantly reduce nodule number in the parent cultivar Bragg, but resulted in 59, 44, and 57% reductions for nts 382, 1007, and 1116. Nodule dry weights were not as severely affected. Increasing the NO_3-N level to 300 mg/pot, further reduced nodule number and dry weight for nts 382 and 1116, but not for nts 1007. At 450 mg NO_3-N/pot, nodulation of all genotypes was almost completely inhibited. Mutant nts 1007 fixed the greatest amount of N_2 at the 7.5, 150, and 300 mg NO_3-N levels. At the 7.5 mg level, nts 1007 fixed 29% more N_2 than Bragg. At the 300 mg level, nts 1007 fixed more than twice as much N_2 as nts 382 or 1116. Nodulation of all the nts mutants was sensitive to the levels of NO_3 used in these experiments. However, nts 1007 showed greater tolerance to NO_3 and supported a higher level of N_2 fixation.

G-51

Supernodulation mutants in Elgin 87 soybean. R.I. Buzzell, B.R. Buttery* and G. Ablett, Agriculture Canada, Research Station, Harrow, Ontario and Ridgetown College of Agricultural Technology Ridgetown, Ontario, Canada.

Elgin 87 soybean (Glycine max) seeds were treated with ethylmethane sulfonate at 0.04M after soaking for 12 hr in water. Field grown M_1 plants were harvested individually. Ten M_2 seeds of each of 999 plants were planted in vermiculite/perlite culture with 14mM combined N. Five M_2 families with supernodulation mutants were identified. Screening of each of these five families for supernodulated plants indicated that the seeds had a genetically effective cell number of about four when treated with EMS. Results from backcrosses of the mutants to Elgin 87 indicate that two loci may be involved in mutant expression with mutation probably occurring at one locus and with Elgin 87 being heterogeneous for dominant and recessive alleles at the other locus. Additional progeny tests and allelism tests are being carried out.

G-52

Mutations for nodulation which also affect the endomycorrhizal symbiosis in Pea (*Pisum sativum* L.). J.P. Guillemin*, G. Duc, A. Trouvelot, V. Gianinazzi-Pearson and S. Gianinazzi. Station de Génétique et Amélioration des Plantes, INRA, BV 1540, 21034 Dijon Cédex, France.

Pea mutants for nodulation were obtained out of two mutagenesis programs using EMS (1,2). Their phenotype was either the absence of nodules (nod-) or the presence of inefficient and white nodules (nod+, fix-). These mutants were submitted to a controlled inoculation with two endomycorrhizal fungi (*Glomus intraradices* and *G. mosseae*).

All 21 (nod+, fix-) mutants presented patterns of mycorrhizal infection, similar to that observed in the control pea lines (myc+) with both fungi. Out of 45 (nod-) mutants, 21 were characterized as resistant to endomycorrhization (myc-), the symbiosis being blocked at an early stage after appressorium formation. The other (nod-) mutants had a myc+ phenotype.

Genetic analyses suggest common loci control simultaneously the two symbioses.

1) K.C. Engwild *et al.*, 1987. Theor. Appl. Genet., 74, 711-713.
2) G. Duc *et al.*, 1989. Plant Science, 60, 207-213.

G-53

Two dimensional polyacrylamide gel analysis of total leaf protein from wild type and supernodulating mutant of soybean. R. Prabhu*, L. A. Sayavedra-Soto and P.M. Gresshoff. Plant Molecular Genetics, Institute of Agriculture and Center of Legume Research, The University of Tennessee, Knoxville, TN 37901-1071 USA

A two dimensional polyacrylamide gel analysis was performed on total leaf protein from wild type and supernodulating mutants of soybean (*Glycine max* (L.) Merr. cv. Bragg). Leaves were investigated because of the reported involvement of the shoot in the control of autoregulation in soybean (1). The total leaf protein from the supernodulating mutant nts382 could be resolved into 170 polypeptides when detected by silver staining. Upon inoculation with *Bradyrhizobium japonicum* USDA110, the one dimensional total protein pattern changed with time in both wild type Bragg and nts382. Using the crosslinker bis-acrylamide in the first dimension, SERVALYT 3-10 Iso-Dalt ampholytes gave a lighter background compared to Pharmalyte 3-10 and equilibration of the first dimension tubes with running buffer before the second dimension SDS-PAGE, minimized streaking. For the mini-gels, freezing the tube gels at -20°C and thawing before the second dimension, produced a complete loss of resolution. This study looks at total existing leaf proteins and is a parallel study to the investigation of *in vitro* translation products by Sayavedra-Soto *et al.* (see this Congress)

(1) Delves, A.C., A. Mathews, D.A. Day, A.S. Carter, B.J. Carroll, and P.M. Gresshoff (1986) Plant Physiol. 82:588-590

G-54

Differences in leaf proteins in soybean (*Glycine max* cv. Bragg) and in the supernodulating soybean mutant (*nts*382). L.A. Sayavedra-Soto*, S.A. Angermüller and P.M. Gresshoff. Plant Molecular Genetics (OHLD). The Institute of Agriculture and Center of Legume Research. University of Tennessee. Knoxville TN 37901.

The regulation of nodulation of soybean is controlled by a feedback response in the leaves (1). Leaves of soybean (*Glycine max* cv. Bragg) and its supernodulating EMS induced mutant *nts*382 (2) were compared for the expression of proteins. Plants were grown in pouches, in a mixture of sand-vermiculite, or in vermiculite and watered with a modified Hoagland nutrient solution (3) supplemented with 0.5 mM nitrate (added to permit healthy development of uninoculated plants, yet not sufficiently high to prevent nodulation). Total RNA and polyA$^+$ RNA was isolated from inoculated and uninoculated plants at selected time points. *In vitro* translated polypeptides were compared by 2-D gel electrophoresis. Protein differences in leaf tissue were detected among the different trials that may be correlated to the mutation, to the development or to inoculation between days 7 and 32 from germination. Other differences are attributed mostly to experimental variation. Discussion on why the proteins of leaves might be involved in the normal feedback response regulating nodulation is made.

1. Gresshoff P.M. and Delves A.C. (1986). Plant Gene Research 3:159-206.
2. Carroll B.J., McNeil D.L. and Gresshoff P.M. (1985). Plant Physiol. 78:34-40.
3. Gremaud M.F. and Harper J.E. (1989). Plant Physiol. 89:169-173

G-55 Analysis of the soybean genome using pulsed field gel electrophoresis. R.P.Funke*, L.A.Sayavedra-Soto, and P.M.Gresshoff
Plant Molecular Genetics, Institute of Agriculture and Center of Legume Research, The University of Tennessee, Knoxville TN 37901-1071, USA

We describe a method for preparing intact soybean DNA for digestion by rare hitting restriction endonucleases, and separation of the resulting large DNA fragments using pulsed field electrophoresis (PFGE). Succesful fractionation of the fragments is demonstrated by hybridization to polymorphic DNA restriction fragments from a soybean genomic library. This technique will be used to walk and jump from loosely linked markers towards the gene determining the nts (nitrogen tolerant symbiosis) phenotype in mutants isolated in this lab using EMS mutagenesis.

G-56 **RFLP analysis of symbiotic mutants of soybean.** D. Landau-Ellis*, S. A. Angermüller, L. J. Schuller, L. Sayavedra-Soto, and P. M. Gresshoff. Plant Molecular Genetics (OHLD), Institute of Agriculture, The University of Tennessee, Knoxville, TN 37920-1071, USA

Mutants isolated by EMS mutagenesis of soybean [*Glycine max* (L.) Merr. cv Bragg] possess altered symbiotic phenotypes resulting in either nonnodulation (nod49 and nod139) or supernodulation (nts382 and nts1007). Restriction Fragment Length Polymorphisms (RFLPs) may be used as genetic markers when linkage is established between nodulation phenotype and a particular banding pattern obtained by Southern hybridization. Interspecific crosses were made between the four *G. max* mutants and *G. soja* (PI 468397) in the summer of 1988. The F1 hybrids were confirmed by plant morphology, flower color, root morphology, and RFLPs. One hundred and ten plasmid clones (from Drs. Paul Keim and Randy Shoemaker, Ames, IA) are being used to detect linkage of RFLPs and the mutant alleles. These clones are part of the soybean RFLP map, which comprises about 1800cmo (≈ % recombination). Given the genomic size of soybean as 1.9 X 10^9 bp, this represents about 10Mb DNA per locus. Segregating populations of the F2 recombinant plants will be analyzed for RFLP linkage, gene order, and distance. No RFLPs have been detected between Bragg and the four mutant phenotypes. This methodology can also be used for the mapping of other symbiotically important genes such as those coding for nodulins.

G-57

Molecular analysis of chickpea (Cicer arietinum L.) nodulation mutants. Vijaya Shri Paramadevanahalli* and Thomas M. Davis. Dept. of Plant Biology, the University of New Hampshire, Durham, NH 03824, USA.

We are using heterologous nodulin cDNA clones as probes to look for restriction fragment length polymorphisms (RFLPs) in radiation-induced chickpea nodulation mutants PM233 (non-nodulating), PM665 and PM679 (temperature-sensitive nodulation), PM405 and PM638 (white, non-fixing nodules), and PM796 (poorly fixing, prematurely senescent nodules) in comparison to their common, wild type parent, ICC 640. The alfalfa nodulin probe A2ENOD2 showed strong, single-band hybridization to Southern blots of chickpea, alfalfa, and soybean genomic DNA cut with EcoRI and Hind III but revealed no RFLPs among chickpea nodulation mutants. Alfalfa nodulin probe 9A did not show hybridization to chickpea, soybean, or alfalfa genomic southerns. Results obtained using other alfalfa and soybean nodulin cDNA probes will be presented.

G-58

Interspecific hybridization in Azolla.L.D.Thanh*,T.V. Tran,N.V.Mui,D.T.Hang,P.K.Trang and N.P.Vy,Dept.of Genetics,University of Hanoi,Vietnam;J.W.Kijne,Dept. of Plant Molecular Biology,Leiden University,Holland.

The crosses among 5 species of Azolla:A.mexicana,A.filiculoides, A.caroliniana,A.microphylla and A.pinnata were made.The reciprocal crosses between A.microphylla and A.caroliniana were successful.

The hybrids CM-588 and MC-588 were confirmed by means of analysis of 6-phosphogluconate dehydrogenase,phosphatase acid and esterase isoenzyme spectrum and of determination of chromosome numbers.The loci controlling isozymes in parents were transferred to the hybrids.The participation of extrachromosomal factors in the formation of hybrids could be excluded.The chromo some numbers of the hybrids were coincident with the expected numbers.The hybrids could be distinguished from their parents by some morphological traits but not so easy.The growth rate and the nitrogen fixing capacity of hybrids were higher than those of the parent materials,about 1.2 and 1.4 times,respectively.The above mentioned hybrids have been applied to production of an area of 800 ha of rice field at 6 agricultural co-operatives of Hanoi during the winter-spring crop 1988-1989. This is the first application of Azolla hybrids on a large scale in agriculture.

G-59

Inter- and intracultivar variation of legumes for the activity of symbiosis with Rhizobium meliloti.

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N_2-fixing (C_2H_2-reductase) activity and symbiotic efficiency (% increase of plant mass) in 99 cultivars of alfalfa (60 - Medicago sativa, 27 - M. varia, 12 - M.falcata), 8 of sweet clover (4 - Melilotus albus, 4 - M. officinalis) and 29 wild and cultivated genotypes of fenugreek (Trigonella foenum graecum) inoculated with 5 different R. meliloti strains were examined. N_2-fixing activity was characterized by lover level of intercultivar variation than symbiotic efficiency. In alfalfa distribution of cultivar mean values for symbiotic efficiency had right-side asymmetry while distribution for N_2-fixing activity had left-side asymmetry. In 6 cultivars of M. varia distribution for N_2-fixing activity of individual plants also had left-side asymmetry while for plants mass decrease of asymmetry coeffi cients after inoculation was observed. Data obtained allow us to suppose that symbiotic efficiency is more strictly controlled by host genotype than N_2-fixing activity. In fenugreek genotypes of Indian subspecies are more susceptible for R. meliloti inoculation than of Mediterranean subspecies (% of nodulated plants was 78 and 20;% of N_2-fixing plants was 61 and 0).

SESSION H

Nitrogen fixation and evolution
H-01 - H-19

H-01 The V-nitrogenase in Anabaena variabilis.

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The occurrence of an alternative, V-containing nitrogenase has been described for the cyanobacterium Anabaena variabilis (Kentemich et al. FEMS Microbiol.Lett. 51, 19 (1988)). Further experiments have shown that:

a) H_2-production is larger in V-grown than in Mo-grown Anabaena cells. H_2-production can be maximized by growing the cells in the presence of EDTA which partially blocks the uptake hydrogenase (Kentemich et al., Naturwiss., in press).
b) Anabaena can only be grown with V under N_2-fixing conditions but not whith nitrate, indicating that a V-containing nitrate reductase does not exist.
c) C_2H_2 supresses H_2-evolution totally in Mo-grown cells but not in cultures which have expressed V-nitrogenase.
d) The cells can also be grown with neither V nor Mo in the medium indicating that a third nitrogenase possibly exists also in Anabaena.

H-02 Regulation of the Vanadium Nitrogenase Structural Genes in Azotobacter chroococcum.

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Azotobacter chroococcum chrosomal mutations in a region bearing homology to, but distinct from, nif EN (Evans et al. 1988) affect N_2 fixation by the vanadium nitrogenase. One of these strains, A. chroococcum MCD-1505, is mutated in an open reading frame displaying some homology to nif A from A. chroococcum and K. pneumoniae, and an even higher degree of homology to vnfA and anfA from A. vinelandii (Joerger et al. 1989). Transcription from the vnfH and vnfD promoters is abolished in MCD-1505, suggesting that the mutated open reading frame encodes a vnf specific transcriptional activator which we call vnfA. Comparison of the putative vnfH and vnfD promoters has allowed us to identify a conserved sequence in the upstream region which may serve as a binding site for vnfA. The regulatory effects of NH_4^+, O_2, V, and Mo on expression of the vanadium nitrogenase will be presented.

D.J. Evans et al. J. Gen. Mib. 134:931-942, 1988.

R. Joerger et al. J. Bact. 171:3258-3267, 1989.

H-03 Factors regulating transcription of the structural genes for Nitrogenase-2 in Azotobacter vinelandii. Susanne Jacobitz* and Paul E. Bishop. Dept. of Microbiology and USDA/ARS, N.C. State Univ., Raleigh, NC 27695-7615 USA.

Azotobacter vinelandii contains three distinct nitrogenases which are expressed under different diazotrophic growth conditions. Nitrogenase-1 is expressed in the presence of Mo, whereas nitrogenase-2 is expressed in the presence of V. A second alternative enzyme, nitrogenase-3, is expressed in its active form when neither Mo nor V are present. In the present study we used lacZ-fusion strains to examine transcription of vnfH and vnfDGK and its dependence on metal availability under diazotrophic conditions. Expression of vnfH and vnfDGK, the structural genes encoding dinitrogenase reductase-2 and dinitrogenase-2, respectively, occurred in the presence of V and in the absence of both Mo and V (conditions under which nitrogenase-3 is expressed). In addition, transcription of both vnfH and vnfDGK occurred in the presence of NH_4^+. This is in contrast to the situation with nifHDK and anfHDGK (the structural genes for nitrogenase-3) where NH_4^+ is observed to repress these operons. Furthermore, transcription of vnfH and vnfDGK are both VnfA dependent, since a VnfA$^-$ mutant showed low β-galactosidase activity under all conditions tested. Transcription of vnfH in the presence of V or in the absence of V and Mo is also AnfA dependent, whereas transcription of vnfDGK seems to be AnfA independent.

H-04 Comparison of the Properties of the Three Nitrogenases from *Azotobacter vinelandii.* Kathleen A. Scorsone, Virginia G. Moore* and Brian J. Hales, Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803-1804, USA.

Azotobacter vinelandii possesses the ability to synthesize three different nitrogen-fixing enzymes, one containing Mo and Fe, another V and Fe and a third that appears to contain only Fe. Properties of component 1 from Fe-nitrogenase, since it contains no metals other than Fe (and Zn), are of special interest. Unlike the component 1 protein from the other enzymes, this protein elutes from DEAE cellulose with a salt concentration greater than that needed to elute component 2. The EPR spectrum of this protein is also unique, possessing no apparent signal arising from an S = 3/2 spin state, a state usually associated with the nitrogenase cofactor center. Although *A. vinelandii* shows good diazotropic growth on Fe, purified Fe-nitrogenase exhibits a low specific activity toward all substrates. Complementation studies suggest that this low activity is primarily a property of component 1.

H-05

The gene encoding Dinitrogenase Reductase-2 is required for expression of the anfHDGK operon in Azotobacter vinelandii.

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Azotobacter vinelandii is capable of expressing three genetically distinct nitrogenases. Nitrogenase-1 is encoded by nifHDK and is expressed only in the presence of Mo. Nitrogenase-2 is encoded by vnfH and vnfDGK and is expressed in presence of V and the absence of Mo. Nitrogenase-3 is expressed under Mo-deficient conditions in the absence of V and is encoded by anfHDGK. Previous results showed that whenever nitrogenase-3 was expressed, dinitrogenase reductase-2 was also present. In the present study we observed that mutants that are unable to synthesize both dinitrogenase reductase-1 and dinitrogenase reductase-2 exhibit an Anf$^-$ phenotype. This was due to the inability of these strains to synthesize nitrogenase-3. An anfH-lacZ fusion strain (constructed with Tn5-B21) was used to monitor transcription of the anfHDGK operon in mutant backgrounds lacking either or both nifH and vnfH. Strains with either nifH and/or vnfH genes accumulated β-galactosidase activity (2298-7535 Miller units) while strains lacking both nifH and vnfH accumulated background levels (176 - 196 Miller units). Since nifH is not normally expressed in the absence of Mo this result indicates that the vnfH gene product is required for transcription of the anfHDGK operon.

H-06

Mutagenesis of the gene coding for a ferredoxin-like protein downstream of the vnfH gene in Azotobacter vinelandii.

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An open reading frame in the same operon, but downstream of vnfH in Azotobacter vinelandii codes for a protein of the following amino acid sequence: MNAIDGYECTVCGDCEPVCPTGSIVFRDDHYAIEADSCNECTD VGETRCLGVCPVDLCIQPLDD. The arrangement of cysteine residues sugests that this could be a ferredoxin-like protein. Does this gene have any role to play in the vnf (vanadium dependent alternative) pathway of nitrogen fixation? More than 1000 Tn5 mutations in the cloned 6 Kb fragment (including flanking regions) were checked, but none could be found in the gene for the ferredoxin like protein. Tn5 could be detected in all other regions, except this 0.3 Kb region. Several mutations were detected in a fragment smaller than 0.3 Kb, so the small size of the fragment was not the limiting factor. Site directed mutagenesis was next done and one base each in the 19th and 20th amino acid codon were changed to generate a unique BglII site. An interposon (BamHI fragment) coding for β-lactamase was inserted into the BglII site. This clone was introduced into A. vinelandii CA12 (Δ nifHDK) and a marker exchange was achieved. The phenotypic character of the resultant mutant would be presented.

H-07

ALTERNATIVE NITROGENASES IN AZOTOBACTER PASPALI. Elazer Fallik[1*], Peter G. Hartel[2] and Robert L. Robson[1]. Departments of Biochemistry[1] and Agronomy[2], University of Georgia, Athens, Georgia 30602, USA

Azotobacter paspali forms a specific nitrogen fixing association with the subtropical forage grass Paspalum notatun (Batatais) (Dobereiner et al, 1972). We are interested in assessing the importance of nitrogen fixation in this association and in developing methods which will allow genetic components of this relationship to be identified. We will present evidence based on hybridization data that both A. paspali ATCC 23397 (wild type strain) and A. paspali ATCC 23833 (type strain) probably contain all three nitrogenase systems (Mo/Fe, V/Fe and Fe/Fe nitrogenases) found in Azotobacter vinelandii. Consequently this system is a potential model in which to study the question of whether alternative nitrogenases can function in natural environments. We have been able to germinate and grow Paspalum notatum (Batatais) in gnotobiotic plant growth assemblies. Wild type and genetically marked strains of A. paspali proliferate in the rhizosphere and produce a 65% increase in plant dry weight in 10 days. The reason for this growth stimulation is not known. We have also been developing genetic systems for A. paspali in order to construct mutants defective in one or more sets of nitrogenase genes. Tn5 transposes in A. paspali and can be used to obtain insertion mutants useful in genetic analysis of the association. Also we report the results of attempts to construct specific mutants in nif and vnf structural genes by gene exchange from created in vitro in Azotobacter chroococcum.

Dobereiner et al, (1972) J. Gen. Microbiol. 71:103-116

H-08

Demonstration of an alternative nitrogenase in Rhodobacter capsulatus. K. Schneider* and A. Müller. Lehrstuhl für Anorganische Chemie I, Universität Bielefeld, D-4800 Bielefeld 1, FRG

In Rhodobacter capsulatus there exists, in addition to a conventional Mo-containing nitrogenase, a second, Mo-independent nitrogenase which was demonstrated in wild type cells as well as in nif HDK$^-$ mutant cells (provided by Dr. W. Klipp, Lehrstuhl für Genetik, Universität Bielefeld). The alternative nitrogenase is repressed by molybdenum. Mo-concentrations > 1 ppb in the growth medium prevented N_2-fixing growth of nif HDK$^-$ cells and the expression of nitrogenase activity. The Mo-independent nitrogenase was optimally derepressed in almost Mo-free media (< 0.05 ppb) which contained high concentrations of iron (0.5-1 mM Fe-citrate) and serine as N-source. Vanadium neither stimulated nor inhibited growth and activity. The most effective methods for the removal of Mo-traces were treatment of the media with activated charcoal and with Mo starved Azotobacter cells. The alternative nitrogenase reduced acetylene to both ethylene and ethane. With whole cells (nif HDK$^-$) the proportion of ethane varied from 2 to 5 % depending on the amount of residual traces of Mo in the medium. The partially purified dinitrogenase (component 1) was analyzed to contain 20 Fe atoms and negligible amounts of Mo, V, W and Re. The reduced dinitrogenase appeared to be completely ESR-silent. The results indicate that the alternative nitrogenase of Rhodobacter capsulatus is not a vanadium-enzyme but rather an Fe-nitrogenase or a nitrogenase with a not yet identified heterometal atom.

H-09

The phylogeny of *nifH:* gene duplication or lateral transfer?

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Does the phylogeny of *nifH* reflect the ancestry of the bacteria that now carry it, or must we postulate that it has been transferred laterally between widely diverged branches of the evolutionary tree? There has recently been a wealth of new molecular data that bear on questions of this kind. Phylogenetic trees for most groups of bacteria are emerging from studies of 16S ribosomal RNA (rRNA) sequences, and these probably represent the evolutionary relationships of the basic bacterial genomes. They can be compared with trees based on *nifH*, for which many published sequences are available, covering a wide range of organisms. On the whole, the two trees agree remarkably well. The main discrepancy is caused by *Clostridium pasteurianum*, which has several *nifH* genes, none of which resembles that of *Frankia*, the only other gram-positive genus examined. Most of the clostridial sequences form a cluster which has no close relatives, but *Clostridium nifH3* clusters with *Azotobacter anfH* (from the Type 3 nitrogenase system) and an archaebacterial gene in a separate grouping that may represent a very ancient duplication event. Indeed, gene duplication and loss, which have certainly occurred during *nifH* evolution, can be invoked to explain all the apparent discrepancies between the trees, as a plausible alternative to long-range lateral gene transfer. Phylogenetic analyses and hypotheses will be presented.

H-10

5S rRNA sequence of Rhizobium meliloti shows identity to "Achromobacter cycloclastes". E.S.Bulygina, T.N.Volkova*, K.M.Chumakov. Institute of Microbiology, USSR Academy of Sciences, 7-2 Prosp. 60 let Oktyabrya, 117811 Moscow, USSR.

We have sequenced, by a modified method of Peatty, the 5S rRNA of Rhizobium meliloti strain 441a (Collection of Institute of Agricultural Microbiology, Leningrad). The sequence turned to be identical to that of a poorly characterized denitrifying bacterium "Achromobacter cycloclastes" IAM 1013 reported by Sh.Ohkubo et al. (J.Biochem., 1986, 100, 1261-1267). This places R.meliloti into the C group of gram-negative denitrifying bacteria, with the closest relationship to Agrobacterium tumefaciens, in good agreement with the clustering obtained using 16S rRNA oligonucleotide signature analysis of fast and slow growing rhizobia and other bacteria of the alpha subdivision of purple bacteria (H.Hennecke et al., Arch.Microbiol., 1985, 142: 342-348). The results are also in accord with the denitrifying activity of R.meliloti. Thus we suggest "Achromobacter cycloclastes" IAM 1013 to be hereafter treated as Rhizobium meliloti.

H-11 16S Ribosomal ribonucleic acid sequence analysis of Rhizobium and Bradyrhizobium strains. T. Gamo, H. Oyaizu* and K. Mori. National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan.*Toyama University, Toyama City, Toyama 930, Japan.

The phylogenetic status of members of the genera Rhizobium and Bradyrhizobium was examined by reverse transcriptase sequencing of 16S ribosomal RNA using approximately 150 strains collected from tropical, sub-tropical and temperate climate zones. Partial sequences for the region between 1220 to 1377 nucleotides could clearly separate the rhizobia into two clusters, Rhizobium and Bradyrhizobium by the sequence of specific 10 nucleotides. Rhizobium could be grouped into three species, viz. R. leguminosarum, R. meliloti(= R. fredii) and R. loti, and 5 new species were identified by substituting some base sequences. Most of the Bradyrhizobium strains isolated from the many leguminous plants showed the same base sequences as B. japonicum except for 7 new species which were identified by substituting some base sequences. Some of these strains were further characterized by physiological properties and cross inoculation experiments.

H-12 **Inter-serogroups diversity of nodulation genes of *Bradyrhizobium japonicum* in Japan and Thailand**
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A total of 252 isolates of *Bradyrhizobium Japonicum* was collected from Japanese and Thai soils(85 from Japan, 167 from Thailand). Genomic DNAs from these isolates and 11 USDA strains were digested with a restriction enzyme *Bam*HI and hybridized to a 32P-labeled 5.4Kb DNA probe of common nod genes(D,A,B,C) from *Rhizobium trifolii*. These isolates were also examined in their serological relatedness by using USDA antisera.

Agglutination analysis indicated that 61 Japanese isolates could be separated into 11 known cross-react groups. While, 85 Thai isolates reacted with USDA antisera. Its react pattern could categorized into 7 known cross-react groups and 10 new cross-react groups. Southern hybridization analysis indicated that each of the isolates had 1 to 8 hybridizing fragments. Hybridizing pattern was the same in the same serogroup except Japanese serogroup 110 and Thai serogroup 6-122-124-129, but greatly different among inter-esserogroups. Hybridization pattern from the 61 Japanese isolates and the 11 USDA strains were classified into 6 markedly different groups. In case of the 85 Thai isolates reacted with USDA antisera, only 2 isolates had the same hybridization pattern with USDA 110. 83 Thai isolates were categorized into 10 hybridization patterns which were unique to Thai isolates.

These results indicate that there are great diversities in nod genes and in serogroups of *Bradyrhizobium japonicum* between temperate and tropical regions.

H-13

Characteristics of two evolutionary lines of *Bradyrhizobium japonicum*. K. Minamisawa*, T. Tsuruta, K. Fukai and S. Onodera. Faculty of Agriculture, Ibaraki University, Ami, Ibaraki 30003, Japan.

A good correlation was found between sequence divergence in and around *nifDKE* genes, rhizobitoxine(RT) production, hydrogen uptake(Hup) phenotype and extracellular polysaccharide(EPS) composition of *B. japonicum*. The sequence divergence in and around *nifDKE* clearly divided *B. japonicum* strains into two different groups, designated genotypes I and II(GTI and GTII). GTI strains produced no RT and an EPS composed of glucose, mannose, (4-*O*-methyl)galactose and galacturonic acid, whereas GTII strains produced RT and an EPS composed of rhamnose and 4-*O*-methyl glucuronic acid. Hup positive strains, possessing the *hup* structural genes, were confined exclusively to GTI. In the presence of GTI and GTII strains, GTII strains exclusively nodulated siratro and Peking, although GTI strains nodulated bred cultivars of soybean better than GTII strains. We also found that GTII strains excrete a large amount of indole-3-acetic acid in cultures.

These results revealed that GTI and GTII strains of *B. japonicum* are two divergent evolutionary lines that define a marked division of various phenotypes. Partial sequences of 16S rRNA suggested that the division between GTI and GTII is a recent evolutionary event as compared with the division between *Bradyrhizobium* and *Rhizobium*.

H-14

Genetic relatedness and taxonomic considerations of *Rhizobium* strains that nodulate *Phaseolus vulgaris* (L.). E. Martínez*, M.A. Pardo, F. Martins, D. Piñero, P. Graham, A. Franco, R. Palacios and L. Segovia. Centro de Investigación sobre Fijación de Nitrógeno, Ap. 565-A, Cuernavaca, Mor.; Centro de Ecología, UNAM, México; CNPBS EMBRAPA Km 47, Rio de Janeiro, Brasil, Dept of Soil Science, University of Minnesota, US.

The taxonomy of *Rhizobium* strains nodulating *Phaseolus vulgaris* (common bean) is at present ill-defined. This may be due to the fact that bean may be nodulated by a wide range of *Rhizobium* strains (1). We have found two types of strains isolated from bean nodules that differ in various genetic markers (2). Type I strains have multiple copies of nitrogenase genes, a narrow nodulation host-range (1) and they share a common pool of plasmidic sequences. Type II strains have single nitrogenase genes and nodulate *Leucaena* species (3). The genetic relatedness of type I strains has been reported (4) and now the genetic relationships of 60 "type II" strains is analyzed by multilocus enzyme electrophoresis. Representative strains have been hybridized to total DNA's, ribosomal DNA's and specific probes from each type. Our results show that type II strains do not belong to the same species as "type I" (*R. leguminosarum* bv. *phaseoli*) strains and may constitute novel species.

1. Martínez, E., *et.al*. J. Gen. Microbiol. 131, 1779, 1985.
2. Martínez, E., *et.al*. Plant & Soil 108, 179, 1988.
3. Martínez, E., *et.al*. J. Bact. 169, 2828, 1987.
4. Piñero, D., *et.al*. Appl. Environ. Microbiol. 54, 2825, 1988.

H-15

Formation of nodule-like structures on the roots of the non-legumes Rice and Wheat. G.L. Bender, L. Preston, D. Barnard and B.G. Rolfe*. Plant Microbe Interaction Group, RSBS, Australian National University, Canberra ACT 2601, Australia.

As a direct result of our research on the genetic basis of the ability of rhizobia to nodulate *Parasponia* (Bender and Rolfe 1985) we were able to engineer a *Rhizobium* strain that occasionally forms nodules on rice plants. This result also follows from earlier work where we were able to induce root hair curling on rice plants using specific *Rhizobium* strains (Plazinski *et al* 1985). At present the frequency of nodulation is very low (about 0.25% of inoculated plants) and unpredictable, but the internal structure of nodules closely resembles that found in legumes and contains membrane encapsulated bacteria.

A range of concentrations of auxins and cytokinins were added to the roots of wheat seedlings. Within a narrow concentration range, the synthetic auxin 2,4-D induced the formation of structures on wheat roots which outwardly resembled nodules formed on legumes. In contrast to rice, the nodules induced by 2,4-D on wheat roots occur on all inoculated plants but the internal structure is not highly organized in the manner of legume nodules. Auxin plus appropriate bacterial strains also induce these structures which are probably modified lateral roots. However, no evidence of nitrogen fixation was ever found with any of these induced nodule-like structures on rice and wheat.

Bender, G.L. and Rolfe, B.G. 1985, Plant Sci. 38: 135-140.
Plazinski, J. *et al* 1985, J. Bacteriol. 163: 812-815.

H-16

Rice root nodules with nitrogenase and hemoglobin. Yuxiang Jing*.Guangshan Li.Xueqing Shan and Jiage Li. Laboratory of Nitrogen Fixation, Institute of Botany, Academia Sinica, Beijing 100044, China

The bacteria of the genus Rhizobium have the ability to establish nitrogen fixing associations with their host legumes. If rhizobia are able to nodulate non-legumes, especially, the monocotyledonous plants and to fix nitrogen, undoutedly, it is one of the ultimately strategical aims in the research on symbiotic nitrogen fixation and paid great attention to by the researchers. We have obtained rice root nodules by inoculation of mutagenized Rhizobium sesbania. The morphology of the rice root nodules is similar to that of Sesbania cannabina and soybean etc.The fine structure of the nodules also has the similarity to that of the legume nodules, such as infection threads and bacteroids. The nodules contain a small amount of nitrogenase with the indication of acetylene reduction 204 nmole/g nodule/h.Western blot assay preliminarily indicated that the nodules maintain a little bit of Mo-Fe protein of nitrogenase and a significant amount of hemoglobin. This implies that the rice host cells should have the conditions(including the hemoglobin synthesis) enough to satisfy the expression of rhizobium nif genes.This exciting results show that the research on symbiotic nitrogen fixation of non-legumes by rhizobia is of expectation and bright prospectiveness in future.

H-17 Response of rice to inoculation with diazotrophic bacteria. C. Zhang*, M. Lim, J. Li, W. Song, S. Ping, H. Wang and C. You, Inst. for Application of Atomic Energy, CAAS, P.O.Box 5109 Beijing100094, P.R. China

5 species of nitrogen-fixers: Alcaligenes faecalis(Af), Klebsiella planticola(Kp), K. oxytoca(Ko), Enterobacter cloacae(Ec) and Azospirillum brasilense(Ab) have been selected as inoculants for eight varieties of dryland and wetland rice(Indica and Japonica). The results indicate that the different species may have a significant responses e.g. Af for some varieties of both Indica and Japonica rice, Ab Sp245 and Kp DWUL2 for Japonica rice, and Ec 26 for Indica rice. Field experiments also confirmed this results, particularly the Ec (pCK_3)-a transformant was more effectively, by using N-15 dilution technique. The root exudates of rice have been examined. The components and contents of root exudates were dependent on the varieties of rice. About 9 kinds of amino acids, 2 kinds of phytohormones namely IAA and GA_3 and malic acid were found in pure culture of Af. The inoculation with Af stimulated the excretion of rice root and affected the components and contents of root exudates. 2.63% of photosynthate in uninoculated rice were excreted, whereas 3.52% was released from inoculated rice. Most of them were uptaken by Af in rhizosphere. The photosynthatic rate and excretory ability affected the nitrogenase activity. The beneficial effects of these bacteria on rice fall into 2 categories-growth promotion and nitrogen fixation.

H-18 Enzyme-induced nodulation of Brassica napus by Rhizobium. E.E. Benson*, M.K. Al-Mallah, M.R. Davey and E.C. Cocking. Department of Botany, University of Nottingham, Nottingham NG7 2RD, U.K.

Enzymatic treatment of the roots of clover seedlings removes the barrier to Rhizobium-host plant specificity (Al-Mallah et al, 1987, Bio/Technology, 5, 1319-1322) and enables the formation of nodular structures on the roots of rice (Al-Mallah et al, 1989, J. Exp. Bot., 40, 473-478) and wheat (Al-Mallah et al, 1989, Int. J. Genet. Manip. in Plants 5, 1-7). Root nodules have also been induced on seedlings of Brassica napus after treatment of the roots with cellulase YC and pectolyase Y23, followed by inoculation with a mixture of R. loti and Rhizobium strain 32H1. Six percent of the seedlings of the cv Rafal nodulated on nitrogen-free agar medium supplemented with 0.1% sucrose. Nodules which developed on the roots of Brassica seedlings were morphologically similar to nodules induced on Trifolium repens by R. trifolii. Brassica nodules showed low nitrogenase activity as determined by acetylene reduction, and tellurite reduction as visualised by electron microscopy. Since plant growth influences nodulation and nitrogenase activity in the legume-Rhizobium symbiosis, the effects are being investigated of nitrogen status, metabolites, vitamins, cofactors, the nature of the root environment and light on Brassica nodulation. Other rhizobia, including those which nodulate the non-legume Parasponia, are also being assessed.

H-19 Inoculation of non-host plants with Frankia. B. C. Mullin and C. S. An*. Department of Botany and The Center for Legume Research, The University of Tennessee, Knoxville, TN 37996-1100, USA, and The Botany Department, Seoul National University, Seoul, Korea.

The actinomycete Frankia is capable of participating in symbioses with plants from at least seven different orders. Although specificity does exist, host range groups frequently include host plant genera from more than one order of plants. Unlike rhizobia which have no intrinsic oxygen protection mechanism, Frankia strains are able to adapt remarkably well to a variety of host plant environments because of their ability to regulate oxygen diffusion into the nitrogen-fixing vesicle. For this reason we believe that frankiae would be more amenable to induced symbioses than rhizobia. Following the observation by Al-Mallah et al. (1989) that rice seedling roots treated for a short time with cellulase and pectinase formed nodular structures in response inoculation with Rhizobium, we have initiated such studies using Frankia as an inoculum. The results of these inoculation tests will be presented.

LIST OF AUTHORS

A

Abe, M. E-21
Ablett, G. G-51
Agibetov, K.A. G-06
Agron, P. L-37
Ahlborn, B. B-16, *B-35
Ahmed, B. *F-01
Alameda, M. B-36
Al-Ani, F.Y. *D-30
Albrecht, S.L. *D-34
Al-Dulaimi, H.M. D-41
Alfano, J.R. *E-06
Al-Ithawi, B.A. D-41
Al-Khafagi, I.A. D-30
Allen, T. G-16
Al-Mallah, M.K. H-18
Al-Rashidi, R.K. *D-06
Al-Sultani, A.K. D-30
Amara, D.S. *F-38
Amarger, N. *E-16
Amijee, F. F-39
An, C.S. *H-19
Andreeva, I.N. *F-23
AngermHller, S.A. G-54, G-56
Anthamatten, D. *E-35
Antoine, A.D. E-110
Aparicio-Tejo,P.M. *C-20
Apte, S.K. *D-08
Araujo, R.S. *B-29, B-30, D-13
Arena, L.M.R. F-02
Arnold, W. E-23
Arp, D.J. *L-27, C-10
Arrarás, E. F-10
Arrese-Igor, C. C-20, G-33
Arunakumari. A. E-95
Ashby, G.A. A-06, A-07
Atherly, A.G. *E-01
Atkins, C. C-19
Attia, M. D-39
Aziz, N.Y. D-06

B

Bach, A.N. E-57, F-40
Badawi, A.M. F-18
Badr El-Din, S.M.S. D-39
Baker, D.D. *D-33
Balatti, P.A. *B-39
Baldani, J.I. E-22
Bali, A. E-92, *E-93
Barbour, W.M. *E-39
Barnard, D. H-15
Barnes, D.K. C-34
Barrilleaux, P.S. A-15
Bassam, B.J. *G-44
Battisti, L. *B-34
Bauer, W.D. B-12
Baune, W. D-23
Beattie, G.A. *B-31
Becana, M. *C-24
Beck, D. D-37
Bedmar, E.J. *E-55
Belavina, N. *F-09
Belefant, H. *G-22
Belen, R.E. F-15
Belli, M.L. D-16
Bender, G.L. H-15
Benet, G. B-06
Benson, D.R. E-82
Benson, E.E. *H-18
Berg, H. *G-08
Bergersen, F.J. *L-24, *C-26
Bergman, B. G-07
Bergström, J. *A-24
Bernardo, M. A-16
Berry, A.M. E-78, *E-79
Beynon, J.L. E-09
Bhagwat, A.A. *B-32
Bhat, U.R. B-21
Birkenhead, K. E-12
Bishop, P.E. *L-51, H-03, H-05
Bisseling, T. *L-49, G-15
Blanchard, C. A-15
Blum, L. D-40
Boddey, R.M. *F-22
Boesten, B. *D-53, E-12
Böger, P. A-14
Bohelto, G.R. F-03
Bohlool, B.B. B-38, *G-38
Boiardi, J.L. F-10
Bona, S. *F-29
Bonam, D. *C-11
Borghese, R. *E-65
Borisov, A. G-14
Boron, L. G-24
Borthakur, D. B-38
Botelho, G.R. F-03
Bothe, H. C-12, *H-01
Botsford, J.L. *E-14
Bott, M. L-22
Bottomley, P.J. *L-28, *D-18, D-19
Bravo, A. E-58, E-60
Breil, B.T. *D-21
Brewin, N.J. *L-19, *B-22, B-23, G-01
Brigle, K.E. A-03, A-04
Brodrick, S.J. *F-39
Brom, S. E-20
Brostedt, E. *E-70
Bruderer, T. E-34
Buck, M. *L-36, *E-90
Buckner, D. B-09
Buikema, W.J. *E-105
Bulygina, E.S. H-10
Burgess, B.K. *L-05, A-01, A-22
Burke, S. A-05
Burleigh, S.H. D-47
Burris, R.H. E-74
Bursakov, S.A. F-40

Busi, L. F-02
Buttery, B.R. *G-51
Buzzell, R.I. G-51

C

Caces, M.L.F. *B-38
Caetano-Anollés, G.G-45, G-46, G-47, *G-48
Calderón, J. *E-58
Cannon, F.C. E-09
Cantwell,J.S. *A-02
Cargill, L. B-25
Carlson, R.W. B-09, B-10, *B-21, F-05, F-06
Carrero, M. *D-22
Cassella, S. *E-62
Caudales, R. *E-110
Cen, Y.H. D-29
Cen, Y-.H. E-31
Chamber, M.A. *D-32
Chan, Y-K. *D-31
Chapman, K. *B-24
Chaudhary, A.H. *D-07
Chaudhary, S.L. *F-16
Chaudri, A. D-24
Chawla, A. C-33
Chen, H. D-11
Chen, J.-S. *L-54, E-102
Chen, W. D-28
Chen, Y.P. *C-35
Chesnokova, O.N. E-03
Chiurazzi, M. E-15, E-25
Cho, M.-J. *B-14
Chudek, J.A. G-01
Chumakov, K.M. H-10
Chumakov, M.I. F-08, *F-24
Chun, J.-Y. *E-37
Chun, T.W. A-01
Ciafardini, G. *F-02
Civardi, L. E-97
Cloutier, J. *D-48
Cocking, E.C. *L-57, H-18
Collet, T.A. A-10, *A-11
Colonna-Romano, S. E-23
Condon, S. D-53
Connolly, E. E-45
Cooney, C.L. A-21
Copeland, L. E-52, *G-36
Coucouvans, D. *L-11
Cournoyer, B. *E-81, E-84
Crank, S. *E-42
Cresswell, A. C-01
Culley, D.E. E-108

D

Dalton, D.A. *G-35
Danso, S.K.A. G-50
Das, H.K. *H-06
Datta, D.B. *G-09
Dávalos, A. E-19
Davey, M.R. H-18
Dávila, G. E-20, E-24
Davis, T.M. B-13, G-57
Dawson, J.O. *D-47, D-51
Day, D. *L-18, C-15, E-46, E-52, *G-29
Dazzo, F. L-16, B-17, *B-19, B-24, B-25
Dean, D.R. *L-53, A-03, A-04, A-13, E-102
De Bruin, F.J. *L-04, C-06, E-47, G-10, G-11
De Faria, S.M. *D-38
De Felipe, M.R. *G-05
Defez, R. E-15, E-25
Degenhart, N.R. C-34
De Las Pe:as E-19
De Ley, J. *D-09
Delgado, M.J. E-55
Del Gallo, M. *F-20
De Lima, H.C. D-38
Delledonne, M. *E-96, E-97
De Lorenzo, C. G-05
Del Valle, R. E-80
De Maagd, R.A. B-20, *E-17
De Mallorca, M.S. D-17
Dénarié, J. *L-15
Denardin, N.D. D-10
Denison, R.F. *C-16, C-18
De Paula, M.A. F-21
De Vries, W. C-06
Diaz, A. C-20
Diaz, C.L. G-41
Diaz del Castillo, L. *C-17
Di Bonito, R. *D-16
Dickstein, R. G-15
Diéguez, R. F-10
Ding, Z. C-07
Ditta, G. *L-37
Djordjevic, M.A. D-11
Döbereiner, J. E-99, F-21, F-22
Dobritsa, S.V. *E-85
Dobson, A. E-12
Dörffler, M. E-64
Douka, C.E. *D-01
Downie, J.A. *L-17, *E-18
Drevon, J.J. *C-02
Dreyer, D.A. B-23
Dua, I.S. *D-04
Dubrovo, P.N. C-08
Duc, G. G-52
Dunn, K. *G-16
Duport, C. A-23

E

Eady, R.R. *L-08
Eaglesham, A.R.J. L-56, C-05
Economou, A. L-17, E-18
Ellis, J.M. L-56, *C-05
Elmerich, C. E-88, E-98
El Mokadem, M.T. *F-18
Emerich, D.W. *L-45, *E-50, G-30
Emtsev, V.T. *F-07, F-08
Encarnación, S. E-60
Ernst, A. *A-14

Eskew, D.L. *G-50
Espin, G. E-15, *E-25
Essa, T.A. *D-41
Estabrook, E. *G-28
Evans, R.W. L-56
Evstigneeva, Z.G. E-57
Eweda, A. D-39

F

Fabbri, P. F-20
Fallik, E. H-02, *H-07
Farnham, M.W. *C-34
Faucher, C. L-15
Favelukes, G. *B-26, B-27, *F-10
Fedorova, M. G-14
Fedulova, N.G. E-56
Feldman, B.J. A-02
Felix, G. G-10
FengZhang, Z. C-07
Ferber, D.M. *C-03
Ferdinandy-van Vlerken, M. C-06
Fernandes, T.A. D-08
Fernando, S.M. *C-19
Figueroa, P. F-10
Filatov, A. G-14
Fischer, H.M. L-22, *E-34
Fisher, K. A-06
Fleischman, D.E. L-56
Flores, M. E-20
Flory, J. A-08
Flynn, K.J. D-25
Fogher, C. E-96, *E-97
Fomina-Eshchenko, J.G. E-04
Forde, B.G. G-25
Foster-Hartnett, D. E-68
Franco, A. H-14
Frank, P. A-02
Freeman, J. G-25
Fritsche, S. E-34
Frolova, V. F-09
Fudi, L. E-29
Fukai, K. H-13
Funayama, S. E-100
Funke, R.P. *G-55

G

Gallon, J.R. *D-25
Gamo, T. H-11
Gandeza, N.E. F-15
Gao, M. E-49
Garcia, V.U. F-15
Garcia-Plazaola, J.I. C-20
Garg, N. D-04
Gartner, E. D-11
Gatta, L. D-16
Gauna, A. F-10
Geelen, D. *E-48, E-49
Genina, O.S. G-06
George, T.A. *A-20
Georgiadis, M.M. *L-09
Gerahty, N. *G-45
Gewaily, E.M. *F-34
Gheller, S.F. A-02
Giacomini, A. D-53
Gianinazzi, S. G-52
Gianinazzi-Pearson, V. G-52
Giller, K. *D-24, E-42, F-39
Gillis, M. D-09
Gilles-Gonzalez, M. L-37
Giménez, M.C. B-26
Girard, M.L. E-20, E-24
Gloux, K. E-61
Godfroy, A. C-02
Goethals, K. E-48, *E-49
Goetting-Minesky, P. L-50
Golden, J.W. *E-106
Golvano, M.P. *G-34
Gonzalez, R. E-14
Goosen-de Roo, L. *B-20
Gordon, A.J. *G-12, G-33
Gormal, C. E-90
Govezensky, D. E-86
Gräfin Schmettow, M. *G-42
Graham, E.T. G-46
Graham, P. H-14
Graham, T.L. B-12
Greener, T. E-86
Gresshoff, P.G.M. G-44, G-45, G-46, *G-47, G-48, G-49, G-53, G-54, G-55, G-56
Groshev, V. F-09
Grunenberg, B. G-11
Gu, B. E-07, *E-08
Gudbrandsen, S.M. D-54
Guerin, V. C-22
Guerinot, M.L. *E-45
Guillemin, J.P. *G-52
Gutkina, A. G-14, G-17
Guy, R. C-04

H

Haaker, H. E-89
Hacin, J.I. G-38
Hai, W.L. E-103
Hales, B.J. A-15, *A-16, A-18, H-04
Hamel, J.-F.P. *A-21
Hamilton, W.D.O. L-17, E-18
Handelsman, J. B-29, B-30, B-31, D-13
Hang, D.T. G-58
Hankinson, T.R. D-54
Hardin, M.G. C-35
Hardy, R.W.F. *L-56, C-05
Harper, J.E. B-14, F-28
Harris, G. A-08
Harris, S. *E-76
Hartel, P.G. D-36, H-07
Hartwig, U.A. B-01, *B-02, B-04
Haselkorn, R. *L-52, E-105

Hashem, F.M. F-32
Hata, S. G-13
Hattermann, D.H. *E-40
Haverkamp, G. H-01
Haylett, T. G-40
Heard, J. *B-13
Hedman, B. A-02
Heichel, G.H. *F-25
Heikkilä-Kallio, U. E-27
Helinski, D.R. L-37
Henjum, K.I. F-25
Hennecke, H. *L-22, E-34, E-35, E-67
Hernandez, A. C-20
Herzog, B. E-34
Heuer, B. *D-03
Hickman, A.B. *A-10, A-21
Higashi, S. *E-21
Hilger, A.B. D-45
Hilgert, U. *C-06
Hirel, B. G-20
Hirsch, A.M. E-83, G-15
Hodgson, K.O. A-02
Hoelzle, I. *C-29
Hollingsworth, R. L-16, *B-19
Holsters, M. E-48, E-49
Honeycutt, R.J. E-01
Hooper, A.B. *L-30
Hoover, T. *E-75, E-87
Hooykaas, P.J.J. G-41
Hopkins, D. A-21
Hopper, S.R.S. *E-104
Hoque, M.S. *F-11
Hoste, B. D-09
Howard, K. A-11
Howes, B.D. A-06
Huakui, C. E-29
Hughes, D.L. A-19
Hungria, M. L-56, *B-03, B-04, C-05, C-30
Hunt, S. C-17, *C-18, C-19
Hunter, J. A-07
Hunter, W.J. *E-54
Hurek, T. D-09, E-101
Hussain, I. *F-37
Huss-Danell, K. D-43, *G-07
Hynes, M.F. *B-33

I

Iaccarino, M. *E-15, E-25
Iismaa, S.E. *A-22
Imai, H. H-12
Imsande, J. *F-30
Ishac, Y.Z. *D-39
Ismailov, S.F. C-08, F-23 ,G-06
Ivanoushkin, A.G. E-57
Ivanov, B.F. E-56
Iyer, R.K. E-109
Izaguirre-Mayoral, M.L *D-17

J

Jacobitz, S. *H-03
Jacobson, M.R. *A-13
James, E. *G-01
Jensen, G.M. A-22
Jha, D.K. *D-46
Ji, L. C-25
Jing, Y. *H-16
Jochimsen, B. *G-21
Joerger, R.D. H-05
Johnson, J.L. E-102
Johnson, M.K. A-15
Johnston, A.W.B. L-17, E-18
Jones, A.D. E-79
Joseph, C.M. *B-01, B-03, B-04
Joshi, P.A. G-45, *G-46, G-47
Joshi, C.P. G-19
Jouanneau, Y. A-23
Julius, C. A-07
Junchu, Z. E-29

K

Kadyrova, M. E-30
Kahn, D. L-37
Kahn, M.L. E-06, E-59
Kaijalainen, S. E-27
Kalakoutsky, K.L. F-40
Kalderon, E.F. E-57
Kalembasa, D.T. F-05, *F-06
Kalembasa, S.J. *F-05, F-06
Kannaiyan, S. *F-13
Kannenberg, E.L. B-22
Kape, R. *B-11
Kapulnik, Y. *B-06, B-18, D-03
Kapustka, L. D-23
Kapuya, J. G-50
Karr, D.B. *G-30
Kastelein, M. B-06
Kaul, B.B. A-20
Kaur, A. *G-33
Keefe, R.G. *C-32
Keister, D.L. B-32
Kelly, M. *E-91
Kennedy, C. E-91, *E-92, E-93
Kent, H.M. E-90
Kentemich, T. *C-12, H-01
Kerber, L.L. *H-02
Kersters, K. D-09
Khailova, G. *D-02
Khan, M.F. F-34
Khan, M.M.A. *B-12
Kheder, A.K. F-34
Kijne, J.W. *L-14, B-07, B-08, *G-41, G-58
Kim, S. A-01
Kindon, N. A-05
King, B.J. C-18
Kinnback, A. *G-02
Kipe-Nolt, J. D-15
Kishinevsky, B.D. *G-39

Kiss, G.B. G-17
Klingmüller, W. E-94
Klipp, W. *L-39, E-10, E-13, E-67, *E-69
Klucas, R.V. C-24, *C-25
Kneen, B.F. L-25
Knight, T.J. G-26
Knobel, H. E-34
Kondorosi, A. *L-35
Kotb, S.I. F-32
Kouchi, H. C-18, *G-13
Kovárová, D. D-12
Kranz, R.G. E-68
Kretovich, W.L. E-56
Krey, R. *E-10
Kruchinina, N. G-14
Krylova, V.V. C-08
Kulikova, O. G-14
Kullik, I. E-34
Kumar, A. F-31
Kumar, D. *C-13
Kumar, H.D. C-13
Kustu, S. E-75, E-87

L

Ladha, J.K. *L-44
Lagares, A. *B-27
Laguerre, G. E-16
Lakshmi, B. B-21
Lalonde, M. *L-43, E-77
Lamberti, A. E-15
Lammers, P.J. *E-107
Landau-Ellis, D. *G-56
Lang, P. G-34
Langeberg, L. G-35
Larkova, T. D-02
Larsen, K. G-21
LaRue, T.A. *L-25
Law, I.J. *G-40
Layzell, D.B. *L-03, C-04, C-16, C-17, C-18
Ledebur, H. E-07
Lee, C.C. B-34
Lee, K.B. F-19
Legocki, A.B. *G-24
Leigh, J.L. B-34
Lerouge, P. L-15
Le Rudulier, D. *E-61
Leung, K. D-18
Lewis, T. E-14
Li, F. D-28
Li, G. H-16
Li, J. H-16
Li, J. H-17
Li, J.-G. A-01
Li, W. D-28
Li, Y. G-29
Li, Z.Z. *G-49
Liang, J.-H. *E-74
LiangShu, X. *C-07
Lin, C. *D-26
Lin, M. H-17
Lindell, J. G-31
Lindström, K. D-05, E-26, E-27, E-28
Lipsanen, P. E-27
Lisá, L. D-12
Lister, D.L. E-46
Liu, C.C. D-26
Liu, Q. *E-78
Liu, Y. *E-59
Livanova, G.I. *C-08
Liyanage, N.D.L. F-27
Lobo, A.L. L-31
Lodeiro, A. F-10
Lois, A. L-37
Long, S. *L-02
Lopes, C. E-15
Lowe, D.J. *A-06, A-19
Lucas, M.M. G-05
Ludden, P.W. L-06, A-05, E-71, E-72, E-74
Lugtenberg, B.J.J. B-07, B-08, E-17, G-41
Luka, S. *E-38
Lundquist, P-O. D-43
Luque, F. A-17
L'vov, N.P. *F-40

M

Machado, H.B. E-100
Madden, M.S. L-06, *A-05
Mahmoud, S.A.Z. F-12
Maier, R.J. *L-23, C-03, C-32, C-33
Manco, G. E-15
Mandchan, K. F-23
Marcker, K. *L-46
Marinelli, G. F-02
Marsolier, M.C. G-20
Martensson, A. *F-33
Marthur, A.N. F-37
Martin, A.E. A-22
Martin, M. F-17
Martin, P. G-43
Martinez, E. E-60, *H-14
Martinez, L.M. E-58
Martinez-Salazar, J.M. E-20, *E-24
Martins, F. H-14
Masepohl, B. *E-13
Mathur, A.N. F-37
May, H.D. *A-03
Maxwell, C.A. B-01, B-04
Mazin, V. D-02
Mazurier, S. E-16
McGrath, S. D-24
McLaughlin, S. E-107
Meyer, A. E-67
Meyer, C. A-23
Meskiéne, I. G-17
Messens, E. E-48
Meza, R. E-15, E-25
Mian, M.H. *F-14
Miao, G.H. G-19, G-20
Micali, F. *F-17

Mikheeva, L. F-09
Milam, J.R. D-34
Miller, A.-F. *A-09, A-21
Miller, R.W. *C-31
Milner, J.L. *D-13
Minamisawa, K. *H-13
Minchin, F.R. C-01, *C-21, G-01, G-33
Miranda, J. E-19
Mishra, R.R. D-46
Mitchenall, L.A. A-17
Mitronova, T. F-09
Moawad, H. *D-37, D-39
Modi, B. *E-63
Modi, R. E-63
Modi, V. E-63
Moloney, A.H. *C-04
Montasser, M.S. *F-32
Moore, V.G. *H-04
Mora, J. E-58, *E-60
Mora, Y. E-58, E-60
Moreau, R.A. E-79
Moreira, F.M. D-38
Moreno, S. E-15, E-25
Mori, K. H-11
Morris, R.H. A-19
Morrisseau, B.A. E-45
Mort, A.J. G-40
Mortenson, L.E. A-12, C-11
Mosca, G. F-17, F-29
Moshiri, F. C-33
Mui, N.V. G-58
Muller, A. H-08
Müller, H.P. G-42
Müller, P. *E-44
Mullin, B.C. *L-50, H-19, G-22, G-23
Mulongoy, K. *L-41
Mulroy, W.A. B-15
Murakami, T. H-12
Murray, R.J. A-21
Myrold, D.D. *D-45

N

Nadeau, P. D-48
Narváez, N. E-60
Nazaret, S. E-84
Némcová, M. D-12
Neves, M.C.P. *F-03, G-32
Newcomb, E.H. G-09
Newman, J.D. *B-37
Newton, W.E. A-02, A-03, A-04
Neyra, C.A. *E-95
Nguyen, M.T. A-22
Nichol, L. G-08
Nick, G. *E-26
Nielsen, G. E-74
Niemann, E.G. E-99
Nixon, B.T. *E-07, E-08
Noel, K.D. B-28, B-37
Nohara, Y. D-20
Nordlund, S. E-70, E-73
Normand, P. E-81, *E-84
Norris, J.H. *G-15
Novák, K. D-12
Novik, N. D-44
Nuti, M.P. D-53

O

O'Brian, M.R. *E-53
O'Connell, K.P. *B-30
Oelze, J. *E-64
O'Gara, F. D-53, *E-12
Olivares, J. E-55
Oliver, M.E. A-15, *A-18
Onishchuk, O.P. E-03
Onodera, S. H-13
Onyeocha, I. *E-98
Orme-Johnson, W.H. A-08, A-09, A-10, A-11, A-21
Osman, S.F. E-79
Oyaizu, H. *H-11

P

Paau, A.S. *L-40
Pabbi, S. F-35
Pace, V.M. *E-68
Pacheco Basurco, J.C. F-10
Pacovsky, R.S. *C-30
Palacios, R. E-20, H-14
Pankratz, S. B-24
Papin, S. E-107
Paradellis, T. D-01
Paramadevanahalli, V.S. *G-57
Pardo, M.A. H-14
Park, G. F-19
Perniske, M. *B-16
Parsons, R. *C-15
Patriarca, E. E-15
Patterson, N.A. *B-18
Pau, R.N. *A-17
Pawloski, K. E-47
Pedrosa, F.O. *E-100
Pees, E. B-07
Peixoto, R.C. G-32
Perotto, S. B-22
Perry, S.M. D-25
Perticari, A. F-10
Peters, N.K. E-32
Phelps, R.H. F-01
Philip-Hollingsworth, S. *L-16, B-19, B-24, *B-25
Phillips, D.A. *L-26, B-01, B-02, B-03, *B-04, *B-05
Pierrard, J. E-66
Pinero, D. H-14
Ping, S. H-17
Poggi, M.C. E-61
Pombeiro, A.J.L. A-19

Poole, R. E-91
Porcari, R. E-96
Porter, S. E-75
Plaxton, W.C. G-37
Prabhu, R. *G-53
Prasad, B.N. *F-36
Preston, L. H-15
Prevost, D. D-48
Priefer, U.B. *E-23
Promé, J.-C. L-15
Provencio, C. E-107
Provorov, N.A. E-05, G-59
Pueppke, S.G. B-39
Pühler, A. E-10, E-13, E-23
Pushkin, A.V. *E-57
Puvanesarajah, V. B-09, B-10

Q

Quinell, R.G. *E-52, G-29
Quinto, C. E-19

R

Radukina, N.L. *G-06
Rahman, M.M. *F-04
Raina, R. H-06
Ramos, M.L.G. F-03
Räsänen, L.A. *E-27
Ratet, P. E-47, G-11
Raucci, G. E-15
Recourt, K. B-07, *B-08
Reddy, A. *E-83
Red'hina, T.V. F-23
Reding, H.K. *D-36
Reeve, M.P. A-11
Reinholt-Hurek, B. D-09, E-101
Reis, V.M. *F-21
Reporter, M. *D-23
Riccio, A. E-15
Richards, R.L. *A-19
Richter, H.J. *G-27
Riedel, K.-U. E-13
Rigaud, J. C-22
Rigo, L.U. E-100
Riou, N. E-61
Roakothan, P. *D-35
Robbins, M. G-35
Robert, F.M. B-38
Roberts, D.M. G-18
Roberts, G.P. *L-55, E-72, E-74
Robson, R.L. A-12, H-02, H-07
Roche, P. L-15
Rochefort, D.A. *E-82
Rolfe, B. *D-11, *H-15
Romanov, V.I. *E-56
Romero, D. *E-20, E-24
Ronson, C.W. *L-34, D-54, E-09
Roskoski, J.P. F-26
Ross, P. D-53
Roth, L.E. D-10, G-03
Ruan, X. *E-32
Rumayuntseva, M.L. *E-05
Rumjanek, N.G. *G-32

S

Saano, A. E-26, E-28
Sadowski, M.J. E-43
Salem, S.H. *F-12
Salifoglou, T. A-10, A-21
Salminen, S.O. *C-28
Salzwedel, J.L. *B-17
Sánchez, F. E-19
Sandhu, G.R. D-07
Sangwan, I. E-53
Sanoria, C.L. F-04
Santana, O. E-19
Santero, E. *L-38, E-75, *E-87
Sato, T. *D-20
Sayavedra-Soto, L.A. G-53, *G-54, G-55, G-56
Schatt, E. A-23
Schatters, B. E-59
Schell, J. C-06, E-47, G-10, G-11
Schell, M.G. E-39
Scheres, B. G-15
Schlüter, A. E-23
Schmehl, M. *E-67, E-69
Schmidt, W. *G-43
Schneider, K. *H-08
Schoot Uiterkamp, A.J.M. *L-12, *L-32
Schramm, U. E-69
Schröder, E.C. *B-36
Schripsema, J. B-08
Schuller, L.J. G-44, G-56
Schuller, K.A. G-37
Schultes, A. E-44
Schultz, B.W. B-37
Schultz, F.A. A-02
Schwintzer, C.R. C-23
Scorsone, K.A. H-04
Scott, D.J. *A-04
Seefeldt, L.C. *A-12
Segal, G. E-86
Segovia, L. H-14
Séguin, A. *E-77
Sellstedt, A. *C-09
Sen, D. *E-22
Senaratne, R. *F-27
Sengupta-Gopalan, C. *L-48, G-26, G-27, G-28
Serrano, A. D-32
Sexton, G.L. D-10
Shah, V.K. *L-06, A-05
Shan, X. H-16
Shanmugasundaram, S. E-104
Shantharam, S. *D-52
Sharma, G.D. D-46
Sharypova, L.A. E-03, E-04, E-10
Shen, S.C. E-11

Shen, W.-J. *G-25
Shramko, V.I. E-56
Shuangqi, C. *C-14, D-27
Shumkova, G. D-02
Sidhipraneed, S. E-33
Silvester, W. E-76
Simarov, B.V. *E-03, *E-04, E-05, *G-59
Simonet, P E-84
Singleton, P.W. G-38
Skrdleta, V. *D-12
Smit, G. B-09, *B-10
Smith, B.E. *L-01, E-90
Smith, G.D. C-09
Smith, R. C-19
Sobral, B.W.S. E-01
Sojda, J. E-07
Solheim, B. D-49
Soliman, A. *E-73
Song, S.D. *F-19
Song, W. H-17
Soto, G. *D-40
Spaink, H.P. B-07
Sprent, J.I. *L-13, D-38, G-01
Squartini, A. B-19
Stacey, G. *B-09, B-10, B-21, E-36, E-37, E-38, E-39, E-40, G-03, G-18
Stephan, M.P. E-99
Stephens, P.J. A-22
Stigter, J. *E-47
Stout, C.D. A-22
Stouthamer, A.H. C-06
Strain, S. *D-19
Strauss, S.H. D-45
Streeter, J.G. *C-27, C-28, C-29
Streit, W. D-14, *D-15
Strijdom, B.W. G-40
Stupar, O.S. *D-44
Sugarawa, S. D-20
Sukwatanasinit, S. E-33
Sun, J-H. *C-10
Suominen, L. E-27, *E-28
Sutherland, T.D. G-44
Sutton, E. *G-03
Svenning, M.M. *D-49
Sweeney, P.E. *D-54
Swensen, S. L-50
Szabados, L. G-11
Szczyglowski, K. G-24

T

Taboada, H. E-60
Tajima, S. *L-21, C-18, *E-51
Tak, T. B-07
Tal, S. *A-01
Taller, B.J. *B-15
Talpassayi, E.R.S. D-50
Tandon, S.R. *G-04
Tao, H. *B-28
Temple, S.J. *G-26
Teuber, L.R. B-05
Thanh, L.D. *G-58
Thipayathasana, P. *E-33
Thomann, H. A-16
Thorneley, R.N.F. *L-07, A-06, *A-07
Tiedje, J. *L-33
Tikhonovich, I. *G-14
Timiriazev, K.A. C-08, F-23, G-06
Tittsworth, R. *A-15
Tiwari, D.N. *F-31
Tjepkema, J.D. *C-23
Toffanin, A. E-62
Tran, T.V. G-58
Trang, P.K. G-58
Trinchant, J.C. *C-22
Tripathi, A. *E-94
Tripathi, S.N. *D-50
Triplett, E.W. D-21, D-22, G-09
Trouvelot, A. G-52
Truchet, G. L-15
Tsai, S.M. B-04
Tsuruta, T. H-13
Tuli, R. *E-109
Turpin, D.H. C-19, G-37
Twigg, P. *G-22

U

Uchiumi, T. E-21
Udvardi, M.K. *E-46, G-29
Ueckert, J. *E-99
Umali-Garcia, M. *F-15
Unkefer, P.J. G-26
Urquiaga, S. F-21, F-22

V

Vachhani, A.K. E-109
Valdes, M. *E-80
Van Berkum, P. *E-43
Van Brussel, A.A.N. *B-07, B-08
Vance, C.P. *L-47, C-34
VandenBosch, K.A. B-22, *B-23
Van den Eede, G. E-48, E-49
Vanderleyden, J. *L-42
Van de Wiel, C. G-15
Van Kessel, C. *F-26
Van Montagu, M. E-48, E-49, *E-101
Vargas, A.A.T. *D-10
Vasconcelos, A.C. E-110
Vázquez, M. *E-19
Vereschagin, A.G. G-06
Verma, D.P.S. *G-19, *G-20
Vieille, C. *E-88
Vignais, P.M. *A-23
Vikman, P-A. *D-43
Vivo, A. G-05
Vogel, C.S. *D-51
Volichenko, Yu. G-14
Volkova, T.N. *H-10

Voltan, R. F-29
Vy, N.P. G-58

W

Wall, J.D. E-65
Wall, L.G. B-26
Walmsley, J. E-92
Wang, B. E-103
Wang, C.L. *E-11
Wang, F.-S. *D-28
Wang, H. E-103, H-17
Wang, S-P. *E-36
Wang, S.-Z. *E-102
Wang, Y. E-12
Wang, Z.F. *D-29
Watanabe, I. D-26
Waters, J.K. E-50
Weaver, C.D. *G-18
Weaver, R.W. E-22
Webb, M.A. *G-31
Webb, M.R. A-07
Wedad D-39
Weeden, N.F. L-25
Weinstein, M. L-37
Welters, P. *G-10, G-11
Werner, D. B-11, B-16, B-35, D-14, D-15, G-02, E-44
Wewad D-39
White, T.C. *A-08, A-11
Whorff, L.L. E-106
Wiegel, J. D-36
Wientjens, R. *E-89
Wiest, D.R. E-106
Wijffelman, C.A. B-07
Williams, M.K. *E-09
Williamson, M.S. G-25
Willison, J.C. *E-66
Wilson, K. *E-41, E-42
Wininger, S. B-18
Wirt, H.J. *E-72
Witty, J.F. *L-20, *C-01, C-21
Woehle, D. *E-71
Wolff, A.B. *D-14, D-15
Wolfinger, E.D. *H-05
Wood, E.A. B-22
Woodley, P. E-92
Wright, D.W. A-10, A-11, A-21
Wright, S. B-24
Wu, S. *F-28

X

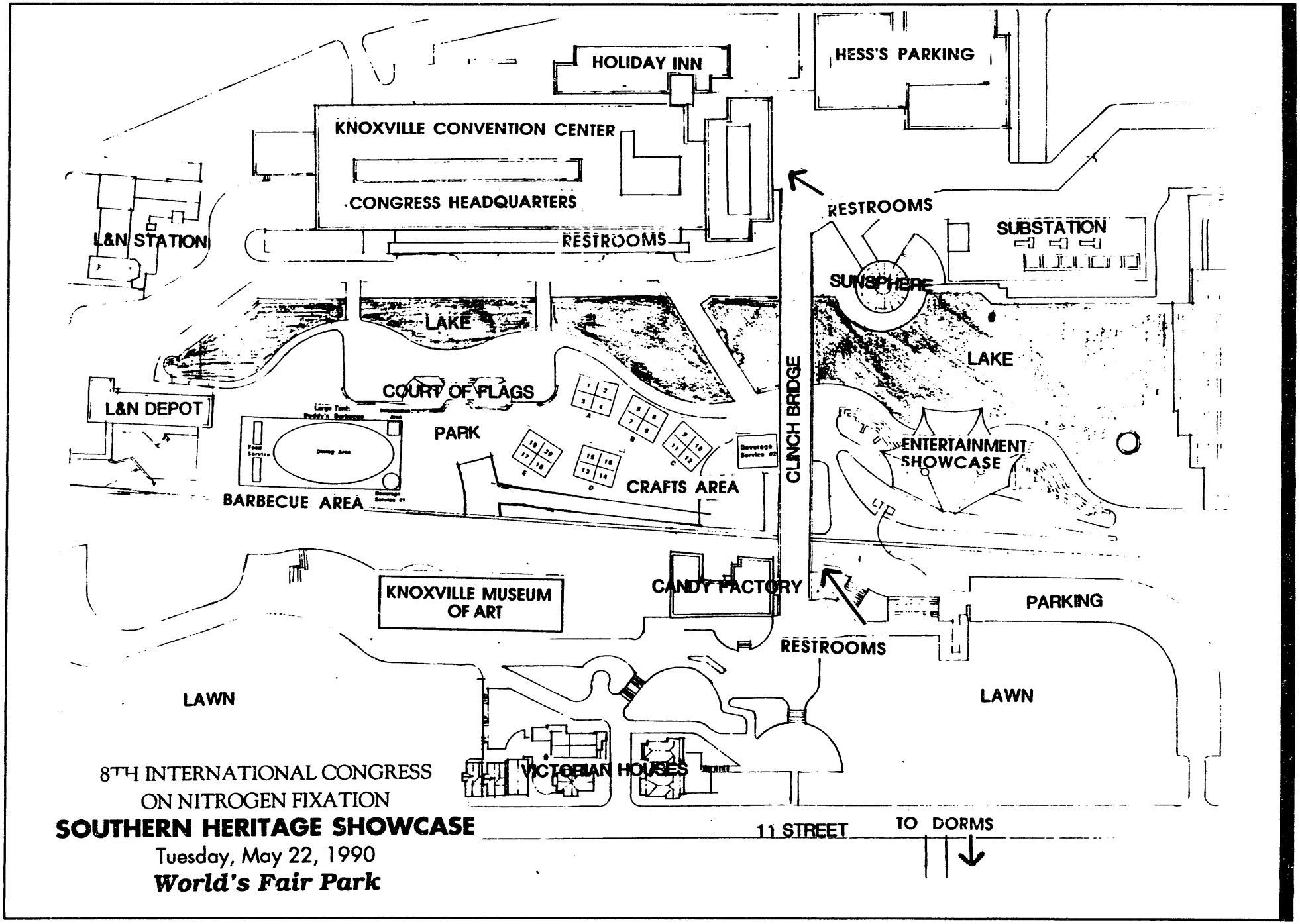
Xenoulis, A.C. D-01
Ximu, M. C-14, *D-27
Xu, H. D-29

Y

Yang, L-J. O. G-29
Yates, G. E-91
Yoch, D.C. C-35
Yokoyama, T. *H-12
Yoneyama, T. *D-42
You, C.B. *E-103, H-17
Young, J.P.W. *H-09
Yu, G.Q. E-02,E-11

Z

Zabelina, J. G-17
Zahran, H.H. D-05
Zamir, A. *E-86
Zammit, A. G-36
Zhang, C. E-103, *H-17
Zhaoping, C. C-14
Zhijian, H. D-27
Zhiznevskaya, G.Ya. G-06
Zhongming, Z. *E-29
Zhou, L.-M. *E-31
Zhu, J.B. *E-02,E-11
Zimmerman, W.J. *E-108
Zinder, S.H. *L-31
Zlotnikov, K. *E-30
Zumft, W.G. *L-29

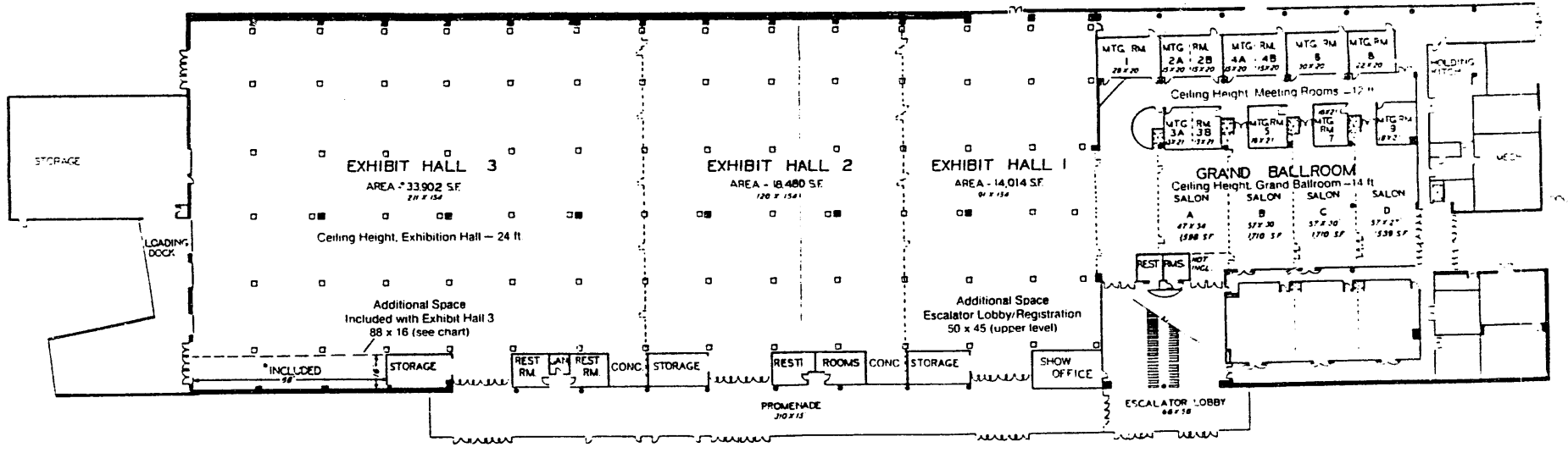


8TH INTERNATIONAL CONGRESS
ON NITROGEN FIXATION

SOUTHERN HERITAGE SHOWCASE

Tuesday, May 22, 1990

World's Fair Park



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KNOXVILLE CONVENTION CENTER

May 20-26, 1990

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