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2. DOE Contract No.
DE-FG05-84ER 13284

3. Title Final Performance Report: Third ASM Conference on Genetics and Molecular Biology of Industrial Microorganisms

4. Type of Document ("x" one)
- ☐ a. Scientific and technical report
- ☒ b. Conference paper:
  - Title of conference Third ASM Conference on Genetics and Molecular Biology of Industrial Microorganisms
  - Date of conference September 30 - October 3, 1984
  - Exact location of conference Indiana University; Bloomington, Indiana
  - Sponsoring organization American Society for Microbiology
- ☐ c. Other (Specify) _____

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8. Submitted by — Name and Position (Please print or type)
Michael I. Goldberg, Executive Director

Organization
American Society for Microbiology

Signature	Phone	Date
Michael I Goldberg	202-833-9680	January 22, 1986

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9. Patent Clearance ("x" one)
- ☐ a. DOE patent clearance has been granted by responsible DOE patent group.
- ☐ b. Report has been sent to responsible DOE patent group for clearance.

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AMERICAN SOCIETY FOR MICROBIOLOGY
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WASHINGTON, D. C. 20006
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DE-FG05-84ER13284

November 11, 1985

Ms. Joyce Carringer
Contract Management Branch
Procurements and Contracts Division
U.S. Dept. of Energy
Oak Ridge, TN 37381

Dear Ms. Carringer:

As directed by the Department of Energy, enclosed are two copies of the Final Performance Report and three copies of the Final Financial Status Report for the ASM Conference on Genetics and Molecular Biology of Industrial Microorganisms which was funded in part by a grant from the Department of Energy.

Two copies of the conference program, abstracts of invited speakers and poster sessions are also enclosed. I am also sending along for your reference a copy of Microbiology 1985 which contains extended abstracts of the symposia presentations.

Again, on behalf of ASM, thank you for supporting us in presenting this highly successful meeting.

Sincerely,

Rita C. Vogler

Rita C. Vogler
Meeting Operations Coordinator

rcv:mob

Enclosure

cc: Richard A. Bray
Richard Franetzki
Michael Goldberg
George Hegeman

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AMERICAN SOCIETY FOR MICROBIOLOGY
OFFICE OF MEETINGS AND EXHIBITS

1913 I STREET, N.W.
WASHINGTON, D. C. 20006
TELEPHONE: CODE 202, 833-9680

FINAL PERFORMANCE REPORT

Third ASM Conference On Genetics And Molecular Biology of Industrial Microorganisms
September 30- October 3, 1984
Bloomington, Indiana
Supported By The U.S. Department Of Energy
Under Grant DE-FG05-84 ER 13284

Under the aegis of the American Society for Microbiology and with the support of the U.S. Department of Energy, the National Science Foundation, and sixteen corporate sponsors, 400 government, corporate, and university scientists met September 30 through October 3, 1984 at Indiana University, Bloomington, for the third ASM Conference on Genetics and Moelcular Biology of Industrial Microorganisms (GMBIM-3).

The conference was comprised of 8 symposium sessions (a total of 34 forty minute talks) and two poster sessions (72 presentations). Topics treated ranged widely over the genetics and biochemistry of numerous microbes of industrial interest and their economically significant products. Genetics and gene expression in Streptomyces constituted a major theme as did expression in Saccharomyces, genetic engineering in fungi and bacteria, and the role of plasmids in encoding genes for pathways for synthesis of novel and interesting products in microbes.

A complete program and abstracts of the symposium and poster sessions were distributed to participants at the Conference and extended abstracts of the symposium presentations were published in Microbiology 1985, pp. 363-479, L. Lieve, ed., American Society for Microbiology, Washington, D.C., (1985).

GMBIM-3, like its predecessors, was deemed successful (see enclosed Conference review, by Dean Taylor, ASM News 51: 135-138, 1985). Because of rapid advances in the areas covered and continuing interest, there will be a GMBIM-4 under similar auspices and in the same location in 1988.

Copies of the conference program, abstracts of invited speakers and poster sessions, and list of registered attendees have been attached for further reference.

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ASM CONFERENCE ON GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL MICROORGANISMS
30 September - 3 October, 1984
Bloomington, Indiana

PREREGISTERANTS LISTING

Alexander, Nancy J., USDA/NRRC, 1815 N. University Street, Peoria, IL 61604

Anagnost, James A., Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003

Anderson, Linda M., Miles Labs, Inc., P. O. Box 932, Elkhart, IN 46515

Armstrong, Katherine, Dow Chemical Co., 1701 Building, Midland, MI 48640

Bailey, David G., U.S. Dept. of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

Ball, Christopher, Panlab Genetics, Inc., 110 - 110th Ave., N.E., Suite 704, Bellevue, WA 98004

Baltz, Richard, Eli Lilly Research Laboratories, 307 E. McCarty Street, Dept. MC627, Indianapolis, IN 46285

Barney, Michael C., Miller Brewing Company, 3939 W. Highland Blvd., Milwaukee, WI 53201

Benzinger, Rolf M., National Science Foundation, Genetic Biology Program, 1800 G Street, N.W., Washington, D.C. 20550

Bernan, Valerie S., Georgetown University Medical School, 3900 Reservoir Rd., N.W., Washington, D.C. 20007

Bibb, Mervyn, John Innes Institute, Department of Genetics, Colney Lane, Norwich NR4 7UH, England

Birmingham, Virginia A., Eli Lilly & Company, 307 E. McCarty Street, Indianapolis, IN 46285

Birnbaum, Jerome, Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065

Blaschek, Hans P., University of Illinois, Dept. of Food Science, 905 S. Goodwin, Urbana, IL 61801

Bok, Song H., Hoffmann-La Roche, Inc., 340 Kingsland Street, Nutley, NJ 07110

Boyer, Ernest W., Miles Laboratories, Inc., Biotechnology R&D, P.O. Box 932, Elkhart, IN 46515

Brawner, Mary E., Smith Kline & French Laboratories, 1500 Spring Garden Street, P.O. Box 7929, Philadelphia, PA 19101

Bromberg, Susan K., Miller Brewing Co., 3939 W. Highland Blvd., Milwaukee, WI 53201

1078400

Brown, Nathaniel H., Schering Plough Corp., 60 Orange St., Bloomfield, NJ 07003

Bruce, Barbara J., The Upjohn Co., Molecular Biology Research 7242-209-7, 301 Henrietta Street, Kalamazoo, MI 49081

Buchanan, Mary L., Miles Laboratories, Inc., 1127 Myrtle St. PO Box 932, Elkhart, IN 46515

Buckingham, Tod A., University of Nebraska, 1407 No. 47th, Lincoln, NE 68503

Burke, Brian E., The Ohio State University, Dept. of Microbiology, 5019 N. High St., Box 29, Columbus, OH 43210

Burnett, J. Paul, Lilly Research Laboratories, 37 East McCarty Street, Indianapolis, IN 46285

Buxton, Frank P., Allelix, Inc., 6850 Goreway Drive, Mississauga, Ontario, CANADA L4V 1P1

Byng, Graham S., Miles Laboratories, Inc., Biotechnology Group, P. O. Box 932, Elkhart, IN 46515

Calcott, Peter H., Dow Chemical Company, CR-Biotechnology Lab, 1701 Building, Midland, MI 48640

Cape, Ronald E., Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608

Cary, Jeffrey W., Louisiana State University, Dept. of Microbiology, 508 Life Sciences Building, Baton Rouge, LA 70803

Cassini, Giovanni R., Lepetit Spa, Via Durando 38, Milano, Italy 20158

Chan, Peter T., Dow Chemical Co., CR-Biotechnology Lab, 1701 Building, Midland, MI 48640

Chang, L.T., Bristol-Myers Co., Bristol-Myers Industrial Division, Box 4755, Syracuse, NY 13221-4755

Chapman, Jerry L., Eli Lilly and Company, 307 East McCarty Street, Indianapolis, IN 46285

Chater, Keith, John Innes Institute, Dept. of Genetics, Colney Lane, Norwich NR4 7UH, England

Chen, Chien, University of Cincinnati, College of Medicine, Dept. of Microbiology & Molecular Genetics, Cincinnati, OH 45267

Chen, Li-Chun, Ohio State University, Dept. of Chemistry, 1043 Jones Tower, Columbus, OH 43210

Chung, Shiau-Ta, Upjohn Company, Fermentation Research and Development Unit, Kalamazoo, MI 49001

Clark, David Worth, Bethesda Research Laboratories, 8717 Grouemont Circle, P. O. Box 6009, Gaithersburg, MD 20877

Coats, John H., The Upjohn Company, Infectious Diseases Research, Henrietta Street, Kalamazoo, MI 49001

1078401

Cohen, Stanley N., Stanford University School of Medicine, Department of Genetics, Stanford, CA 94305

Cole, Sheri P., Purdue University, , W. Lafayette, IN 47907

Coleman, Rebecca H., University of Illinois Urbana-Champaign, Microbiology Dept. 342 Burrill Hall, Urbana, IL 61801

Coleman, Robert D., CPC International, Biotechnology Dept., Moffett Technical Center, Box 345, Summitt-Argo, IL 60501

Cox, Donald P., Janssen Pharmaceutica, 40 Kingsbridge Road, Piscataway, NJ 08854

Cox, Karen L., Eli Lilly & Company, 307 E. McCarty Street, Indianapolis, IN 46285

Crabb, W. Douglas, Genencor, Inc., 180 Kimball Way, South San Francisco, CA 94080

Cruze, John A., Phillips Petroleum Co., 222 CPL, PRC, Bartlesville, OK 74004

Davies, Julian, Biogen SA, 3 Route De Troinex, 1227 Carouge IGE, Switzerland

Day, Lawrence E., Eli Lilly & Co., 307 E. McCarty Street, Indianapolis, IN 46285

Dekleva, Michael L., Ohio State University, Dept. of Microbiology, 484 West 12th, Columbus, OH 43210

DeLorbe, William J., Molecular Genetics, Inc., 10320 Bren Road East, Minnetonka, MN 55343

Demain, Arnold, Massachusetts Institute of Technology, Dept. of Nutrition and Food Science, Room 56-123, Cambridge, MA 02139

DeWitt, Janet P., Abbott Laboratories, D-451, R-5, North Chicago, IL 60064

Dey, Chitta Ranjan, Univ. of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267

Dilts, Judith A., University of South Dakota, Dept. of Microbiology, Vermillion, SD 57069

Dizikes, George, UCLA, 10801 Rose Ave. Apt 10, Los Angeles, CA 90034

Dolan, Kathy M., The Ohio State University, Dept. of Microbiology, 484 West 12th Avenue, Columbus, OH 43210

Donovan, Mary Jo, University of Louisville, School of Medicine, Dept. Microbiology & Immunology, Health Sciences Center, Louisville, KY 40292

Dooley, Thomas P., Indiana University, Dept. of Biology, Jordan Hall, Bloomington, IN 47405

Duncan, Janice S., Ohio State University, Dept. of Chemistry, 140 W. 18th Ave., Columbus, OH 43210

Duncombe, George R., Miller Brewing Company, 3939 W. Highland Blvd., Milwaukee, WI 53201

1078402

Dwyer, Mary T., Dalhousie University, Microbiology Dept., 7th Floor, Sir Charles Tupper Medical Bldg., Halifax, Nova Scotia, Canada B3H 4H7

Eckhardt, Thomas G., Smith Kline and French Laboratories, P.O. Box 7929, Philadelphia, PA 19101

Efthymiou, Constantine J., St. John's University, Dept. of Biological Sciences, Jamaica, NY 11439

Egan, Kevin M., AMGen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320

Epp, Janet K., Eli Lilly & Co., 307 E. McCarty St., Indianapolis, IN 46285

Eveleigh, Douglas E., Rutgers University, Biochemistry & Microbiology, Cook College, New Brunswick, NJ 08903

Fayerman, Jeffrey, Eli Lilly Research Laboratories, 307 E. McCarty Street, Dept. MC627, Indianapolis, IN 46285

Feitelson, Jerald S., American Cyanamid Company, Lederle Laboratories, Pearl River, NY 10965

Feldberg, Larry R., American Cyanamid Company, 697 Route 46, P.O. Box 2088, Clifton, NJ 07015

Fischhoff, David A., Monsanto Co., 800 N. Lindbergh Blvd., St. Louis, MO 63167

Fishman, Scott E., Eli Lilly & Co., 307 E. McCarty Street, Indianapolis, IN 46285

Floss, Heinz G., Ohio State University, Dept. of Chemistry, 140 W. 18th Ave., Columbus, OH 43210

Force, Leonard J., Alerta Research Council/Bio Logicals Joint Venture, 11315 87 Avenue, Edmonton, Alberta, Canada T6E 2CZ

Fujii, Isao, Ohio State University, Dept. of Chemistry, 140 W. 18th Ave., Columbus, OH 43210

Gallo, Benedict J., U.S. Army Research & Development Center, Science & Advanced Technology Laboratory, Natick, MA 01760

Galluppi, Gerald R., Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167

Garcia, Jose L., Antibioticos S.A., Brava Murillo 38, Madrid, SPAIN 28015

Garvin, Robert T., Cangene Corp., 6291 Dorman Rd. Unit #12, Mississauga, Ontario, Canada 14V 1K6

Geiger, Jon R., Olin Research Center, P.O. Box 586, Cheshire, CT 06410-0586

Gibb, Greg D., Ohio State Univesity, 5028 Dierker Road, #B5, Columbus, OH 43220

Gillum, Amanda M., E. R. Squibb & Sons, Inc., P. O. Box 4000, Princeton, NJ 08540

1078403

Gingeras, Thomas R., The Salk Institute Biotechnology/Industrial Associates, P.O. Box 85200, San Diego, CA 92138

Gold, Larry, University of Colorado, MCDB, Boulder, CO 80309

Goldberg, Alfred, Harvard Medical School, Dept. of Physiology and Biophysics, 25 Shattuck St., Boston, MA 02115

Goldberg, Richard B., Merck & Co., Inc., R50G-203, Merck Sharp and Dohme, Rahway, NJ 07065

Golden, Frank J., U.S. Food and Drug Administration, 67 Moore Avenue, Kenmore, NY 14223

Graham, R. Scott, The Upjohn Company, 7242-209-6, Kalamazoo, MI 49001

Grosch, Josephine C., Miles Laboratories, Inc., 1127 Myrtle St. (Bldg. 17), P. O. Box 932, Elkhart, IN 46515

Groves, David, Imperial Chemical Industries, Corporate Bioscience Group (ICI PLC), P.O. Box 11, The Heath, Runcorn, Cheshire, ENGLAND WA7 4QE

Grund, Alan D., University of Wisconsin, School of Pharmacy, 425 N. Charter St., Madison, WI 53706

Guarante, Leonard, Massachusetts Institute of Technology, Dept. of Biology, Cambridge, MA 02139

Guijarro, Jose, Harvard University, The Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138

Guterman, Sonia K., Biotechnica International Inc., 85 Bolton Street, Cambridge, MA 02140

Guyer, Mark S., Genex Corp., 16020 Industrial Dr., Gaithersburg, MD 20877

Haas, Elizabeth S., Ohio State University, Department of Microbiology, 484 W. 12th Avenue, Columbus, OH 43210

Haas, Michael J., U.S. Dept. of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

Hafner, Edmund W., Signal UOP Research Center, 50 UOP Plaza, Des Plaines, IL 60016-6187

Hampson, Brian, University of Illinois, Dept. of Food Science, 905 S. Goodwin, Urbana, IL 61801

Harriman, Philip D., National Science Foundation, 1800 G St., NW, Washington, D.C. 20550

Hartley, Donna L., Medical College of Virginia, Virginia Commonwealth University, MCV Station, Richmond, VA 23298

Hartman, Jacob R., Biotechnology General Ltd., Kiryat Weizmann, Rehovot, ISRAEL 76326

Hauer, Bernhard F., BASF, ZH/BC-A30 BASF AG, Ludwigshafen, FEDERAL REPUBLIC OF GERMANY 6700

1078404

Hayes, Michael V., Glaxo Group Research Ltd., Biotechnology Dept., Greenford Road, Greenford, Middlesex, ENGLAND UB6 OHE

Heden, Lars-Olof, KabiGen AB, S-112 87, Stockholm, Sweden

Hegeman, George, University of Indiana, Biology Department, Jordan Hall, Bloomington, IN 47405

Heim, Jutta M., Ciba-Geigy Ltd., Dept. Biotechnology, U-681.346, Basel, SWITZERLAND CH-4002

Herber, Wayne K., Georgetown University Medical School, 3900 Reservoir Rd, N.W., Washington, D.C. 20007

Herrold, Richard E., Ohio State University, Chemistry Dept., 88 W. 18th Ave., Columbus, OH 43210

Hershberger, Charles L. , Eli Lilly & Company, 307 E. McCarty Street, Indianapolis, IN 46285

Heruth, Dan, University of South Dakota School of Medicine, Dept. of Microbiology, Vermillion, SD 57069

Hess, Gerald F., The Upjohn Company, 7242-209-6, Kalamazoo, MI 49001

Heyneker, Herbert L., Genencor, 180 Kimball Way, South San Francisco, CA 94080

Hill, John C., Northwestern University, Dept. Molecular Biology, 303 E. Chicago, Chicago, IL 60611

Hintermann, Gilberto, Mikrobiologisches Institut, Eth-Zentrum Universitatstr, 2, Zurich, SWITZERLAND 8092

Hirshfield, Irvin N., St. John's University, Dept. Biological Sciences, Jamaica, NY 11439

Hjorth, Rolf RH, BioCell Laboratories HB, S751-82, Uppsala, Sweden

Hoffman, Guy G., University of Wisconsin, School of Pharmacy, 425 N. Charter Street, Madison, WI 53706

Hopper, John B., Ohio State University, Microbiology Dept., 484 W. 12th Avenue, Columbus, OH 43210

Hopwood, David, John Innes Institute, Dept. of Genetics, Colney Lane, Norwich NR4 7UH, England

Hornemann, Ulfert, University of Wisconsin, School of Pharmacy, 425 N. Charter Street, Madison, WI 53706

Horvath, Barbara A., Upjohn Co., 7000 Portage Road, Kalamazoo, MI 49001

Hsu, Charles J., Rohm and Haas Co., 727 Norristown Road, Springhouse, PA 19477

Hu, Wei-Shou, University of Minnesota, 421 Washington Avenue, SE, Minneapolis, MN 55455

1078405

Hughes, Patricia F., Genex Corporation, 16020 Industrial Drive, Gaithersburg, MD 20877

Humphreys, Gwynfor O., Celltech Ltd., Dept. of Microbial Genetics, 250 Bath Road, Slough Berkshire, ENGLAND SL1 4DY

Hussain, Musaddeq, Rutgers University, Waksman Institute of Microbiology, P. O. Box 759, Piscataway, NJ 08854

Hutchinson, C. Richard, University of Wisconsin, School of Pharmacy, 425 N. Charter Stree Madison, WI 53706

Illingworth, Charles A., University of Wisconsin, School of Pharmacy, 425 N. Charter Street, Madison, WI 53706

Ingolia, Thomas D., Eli Lilly and Company, 307 E. McCarty Street, Indianapolis, IN 46285

Jackson, David, Genex Corporation, 16020 Industrial Drive, Gaithersburg, MD 20877

Jackson, Marianna, Abbott Laboratories, D-47P, North Chicago, IL 60064

Jacobson, Gunnard K., Universal Foods Corp., 6143 N. 60th Street, Milwaukee, WI 53218

Jellis, Cindy L., Repligen Corp., 101 Binney Street, Cambridge, MA 02142

Johnson, Charles, Syntro Corp., 10655 Sorrento Valley Road, San Diego, CA 92121

Jolly, Setsuko O., Universal Foods Co., 6143 N. 60th St., Milwaukee, WI 53218

Jones, George, University of Michigan, 2084 Natural Sciences Bldg., Dept. of Biochemistry Division of Biological Sciences, Ann Arbor, MI 48109

Jones, Michael D., Eli Lilly & Co., MC627, 307 E. McCarty Street, Indianapolis, IN 46285

Katz, Edward, Georgetown University School of Medicine, Dept. of Microbiology, 3900 Reservoir Road, Washington, D.C. 20007

Katz, Leonard, Abbott Laboratories, Abbott Park, North Chicago, IL 60064

Keggins, Kathleen M., IGI Biotechnology, 9110 Red Branch Road, Columbia, MD 21045

Keith, Paula M., IMC Corp, Microbiology R & D, Terre Haute, IN 47808

Kendrick, Kathleen E., Ohio State Univesity, Dept. of Microbiology, 484 West 12th Avenue, Columbus, OH 43210

Khalifa, Khalifa Ibrahim, , 867 Beacon Street, Apt. #3, Boston, MA 02215

Kim, Young J., Korea Steel Chemical Co. Ltd., Kolon Bldg 6th Floor, 45 Mugyo-Dong, Jung-Gu, Seoul, Korea

Kinashi, Haruyasu, University of Wisconsin, School of Pharmacy, 425 N. Charter St., Madison, WI 53706

1078406

Kleid, Dennis, Genentech, Inc., Dept. of Vaccine Development, 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Kovacevic, Steven, Eli Lilly and Company, 307 E. McCarty Street, Indianapolis, IN 46285

Krogsrud, Richard L., University of Guelph, Department of Chemistry & Biochemistry, Guelph, Ontario, Canada N1G 2W1

Krohn, Ingrid M., Butterworth Publishers, 80 Montvale Avenue, Stoneham, MA 02180

Krzyzek, Richard A., Molecular Genetics, Inc., 10320 Bren Road East, Minnetonka, MN 55343

Kuhstoss, Stuart A., Lilly Research Laboratories, 307 E. McCarty Street, Indianapolis, IN 46285

Lago, Barbara D., Merck & Co., Inc., P.O. Box 2000, Rahway, NJ 07065

Lampel, Jay S., Ohio State Univesity, Dept. of Microbiology, 484 West 12th Avenue, Columbus, OH 43210

Lampen, J. Oliver, Rutgers University, Waksman Institute of Microbiology, P.O. Box 759, Piscataway, NJ 08854

Lampen, Oliver, Waksman Institute of Microbiology, Rutgers University, P.O. Box 759, Piscataway, NJ 08854

Lancini, Giancarlo, Lepetit Rej. Lab, via Durando 38, Milano, Italy 20158

Larson, Jeffrey L., Eli Lilly & Co., 307 E. McCarty Street, Indianapolis, IN 46285

Lasure, Linda L., Miles Laboratories, 1127 Myrtle St., P.O. Box 932, Elkhart, IN 46516

Lawrence, Ellen B., Monsanto, 800 N. Lindbergh, St. Louis, MO 63167

Lee, Jung J., Ohio State University, Dept. of Chemistry, 140 W. 18th Ave., Columbus, OH 43210

Lein, Joseph, Panlabs, Inc., P. O. Box 190, Deer Harbor, WA 98243

Leitner, Ernst, BIOCHEMIE Gesellschaft m.b.H., A-6250, KUNDL/Tyrol, AUSTRIA

Lessie, Thomas G., University of Massachusetts, Department of Microbiology, Amherst, MA 01003

Lewis, Herman W., National Science Foundation, 1800 G. St N.W., Washington D.C., 20550

Lin, Norm S., Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080

Liu, Chung-Cheng, Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94080

Liu, Ci-Jun, University of Maryland , School of Pharmacy, Rm 547, 20 North Pine Street, Baltimore, MD 21201

1078407

Losick, Richard, Harvard University, Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138

Lotvin, Jason A., Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003

Love, Susan F., Lederle Laboratories Medical Research Division, 96A/207, Pearl River, NY 10965

Lovett, Paul, University of Maryland, Dept. of Biological Sciences, Catonsville, MD 21228

Lucklow, Verne A., Northwestern University Medical School, Dept. Molecular Biology, 303 E. Chicago Ave. S4-490, Chicago, IL 60611

Lydiate, Derek J., John Innes Institute, Dept. of Genetics, Colney Lane, Norwich, England NR4 7UH

Macaluso, Anthony, Massachusetts Institute of Technology, Division of Biological Sciences, University of Michigan, Ann Arbor, MI 48109-1048

Machanoff, Richard, Oak Ridge National Labs, Biology Division, P. O. Box Y, Oak Ridge, TN 37831

MacNeil, Douglas J., Merck & Co., Inc., Microbial Genetics, P.O. Box 2000, Rahway, NJ 07090

Majer, Jaroslav, Northwestern University, Medical & Dental Schools, 303 E. Chicago Ave., Chicago, IL 60611

Makins, John, Bristol-Myers, Fermentation Development, Industrial Division, P.O. Box 657, Syracuse, NY 13201

Malik, Vedpal V., Philip Morris Research Center, P.O. Box 26583, Richmond, VA 23261

Manis, Jack J., The Upjohn Co., Fermentation R&D, 7171 Portage Road, Bldg. 89, Kalamazoo, MI 49001

Marrero, Robert, University of Rochester Medical Center, Department of Microbiology, Box 672, 601 Elmwood Ave., Rochester, NY 14642

Mattes, Ralf, Boehringer Mannheim Corp., Bahnhofstrasse 9-15, Tutzing, West Germany D-8132

Mayford, Mark, University of Wisconsin Medical School, Pharmacology Dept., 1300 University Avenue, Madison, WI 53706

Mayles, Bernard A., Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003

McCormick, Pamela A., Merck & Co., P.O. Box 2000, Rahway, NJ 07065

McDonald, C. Cameron, DuPont, DuPont Exp. Station, Bldg. 402, Wilmington, DE 19898

McGilvray, Derek, Eli Lilly & Company, 307 East McCarty Street, Indianapolis, IN 46285

Meade, James, Cetus Corporation, 1400 Fifty-Third St., Emeryville, CA 94710

1078408

Metz, Raymond J., CPC International, Room 90-414, Moffett Technical Center, Box 345, Summit, IL 60501

Mezes, Peter S., Dow Chemical Company, Central Research, 1701 Building, Midland, MI 48640

Mielenz, Jonathan R., G.D. Searle & Co., Searle R&D Division, Skokie, IL 60077

Miller, Bruce, University of California, Dept. of Plant Pathology, Davis, CA 95616

Miller, James R., Lilly Research Laboratories, MC 627, Molecular Genetics Research 307 E. McCarty Street, Indianapolis, IN 46285

Miller, Timothy J., Norden Laboratories, Inc., P. O. Box 80809, Lincoln, NE 68501-0809

Modi, Hemant N., Illinois Institute of Technology, 967 Stuart Drive, Bartlett, IL 60103

Monticello, Daniel J., Miles Laboratories, Inc., Enzyme Process Development Laboratory, P. O. Box 932, Elkhart, IN 46514

Moon, Hong Mo, N.Y.S. Basic Research Institute, Dept. of Molecular Biology, 1050 Forest Hill Road, S.I. New York, NY 10314

Morgan, Alice E., Dow Chemical Co., 1701 Bldg., Midland, MI 48640

Morosoli, Rolf, Institute Armand-Frappier, 531 Blvd. Des Prairies, Ville de Laval, Quebec, Canada H7N 4Z3

Motamedi, Haideh, University of Wisconsin, School of Pharmacy, 425 N. Charter St., Madison, WI 53706

Motley, Stanley T., The Upjohn Company, 7242-209-623, Kalamazoo, MI 49001

Mumford, Michael B., Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94084

Munayyer, Hannan K., Schering Corp., 60 Orange St., Bloomfield, NJ 07003

Myers, Ronald B., G.D. Searle & Co., 30 Buell Street, Harbor Beach, MI 48441

Nakazawa, Hisayoshi, Georgetwon University, Johnson Avenue, Bethesda, MD 20817

Narasimhan, Meena L., University of Illinois Urbana-Champaign, 131 Burrill Hall, 407 South Goodwin, Urbana, IL 61801

Narva, Kenneth E., Louisiana State University, 508 Life Sciences, Baton Rouge, LA 70803

Neway, Justin O., Genetics Institute, 225 Longwood Avenue, Boston, MA 02115

Nimeck, Maxwell W., Schering Corp., 2000 Galloping Hill Road, Kenilworth, NJ 07033

Nyberg, Kerstin I., University of Helsinki, Recombinant DNA Laboratory, Valimotie 7, Helsinki, Finland 00380

O'Neill, William P., Poly-Planning Services, 1338 Rossway Ct., Los Altos, CA 94022

1078409

Olins, Peter O., Monsanto Co., 800 N. Lindbergh Blvd. (U4D), St Louis, MO 63167

Olsen, Mary K., The Upjohn Company, 7242-209-7, Kalamazoo, MI 49001

Omer, Charles, Stanford University, Dept. of Genetics, S-337, Stanford, CA 94305

Otten, Sharee L., University of Wisconsin, School of Pharmacy, 425 N. Charter St., Madison, WI 53706

Otto, Chris J., University of Wisconsin, 3316 Chamberlain Hall, Madison, WI 53706

Palmer, Donna T., Battelle Memorial Institute, 505 King Ave., Columbus, OH 43201

Panchal, Chandra j., Labatt Brewing Company Ltd., Production Research Department, 150 Simcoe Street, London,, Ontario, Canada N6A 4M3

Parag, Yair, The Hebrew University, Department of Genetics, Jerusalem, ISRAEL 91904

Park, Doo H., University of Arizona, Dept. of Microbiology & Immunology, College of Medicine, Tuscon, AZ 85724

Park, Frances J., Genex Corporation, 16020 Industrial Drive, Gaithersburg, MD 20877

Patt, Tom E., The Upjohn Co., Fermentation R & D, Kalamazoo, MI 49008

Paulus, Thomas J., Abbott Laboratories, D451, Bldg. R5, North Chicago, IL 60064

Penalva, Miguel A., Antibioticos S.A., Bravo Murillo 38, Madrid, SPAIN 28015

Pero, Janice G., BioTechnica International, Inc., 85 Bolton Street, Boston, MA 02140

Phillips, Steven J., Dow Chemical Company, 1701 Pruitt Research Center, Midland, MI 48640

Pidcock, Kenneth A., Lehigh University, Dept. of Biology, Williams Hall #31, Bethlehem, PA 18015

Pilacinski, William P., Molecular Genetics, Inc., 10320 Bren Road East, Minnetonka, MN 55343

Piret, Jacqueline M., Mikrobiologisches Institut, Eth-Zentrum, Universitatstr, 2, Zurich, SWITZERLAND CH-8092

Plocher, Sabine P., Ohio State University, Dept. of Microbiology, 484 W. 12th Avenue, Columbus, OH 43210

Pogell, Burton M., University of Maryland, School of Pharmacy, 20 N. Pine St., Baltimore, MD 21201

Polisky, Barry, University of Indiana, Biology Department, Jordan Hall, Bloomington, IN 47405

Pollack, Sidney, Lebanon Valley College, University Apartments West #110, Bloomington, IN 47401

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Quackenbush, Robert L., University of South Dakota, Dept. of Microbiology, Vermillion, SD 57069

Queener, Stephen W., Eli Lilly and Company, 307 E. McCarty St., Dept. KY412, Indianapolis, IN 46285

Racine, Francis M., Enzyme Technology, 783 US250E, Route 2, Ashland, OH 44805

Randall, Linda, Washington State University, Biochemistry/Biophysics Program, Pullman, WA 99164

Ranes, Monica, Harvard University, The Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138

Rao, Nagaraja, Eli Lilly & Co., 307 E. McCarty Street, Indianapolis, IN 46285

Ratzin, Barry J., Am Gen, 1900 Oak Terrace LANE, Thousand Oaks, CA 91320

Reeve, John N., Ohio State University, Dept. of Microbiology, 484 W. 12th Ave., Columbus, OH 43210

Reinert, William R., Campbell Institute for Research and Technology, P-152 Co. Rd. 12, Napoleon, OH 43545

Reynolds, Bob, Enzyme Technology Corp., 783 Rt 250 East, Rt. 2, Ashland, OH 44805

Reynolds, Patricia A., Eli Lilly & Co., 307 McCarty St., Indianapolis, IN 46285

Rheaume, Lisa J., Dow Chemical Co., 1701 Bldg., Midland, MI 48640

Richardson, Mark A., Eli Lilly & Co., 307 E. McCarty St., Indianapolis, IN 46285

Riley, michael i., University of Kentucky, Department of Biochemistry, Lexington, Kentucky 40536-0084

Rosenfeld, Stuart, American Cyanamid Co., Medical Research Division, Lederle Laboratories, Pearl River, NY 10965

Rosteck, Paul R., Eli Lilly & Company, 307 E. McCarty Street, Indianapolis, IN 46107

Rowlands, Robert T., Panlab Genetics, Inc., 110 - 110th Avenue, N.E., Suite 704, Bellevue, WA 98004

Ruby, Carolyn, Merck & Co., Inc., P.O. Box 2000, Rahway, NJ 07065

Rudd, Brian A. M., Glaxo Group Research Ltd, Biotechnology Dept., Greenford Road, Greenford, Middlesex, ENGLAND UB6 OHE

Ryan, Michael J., Schering Corp., 86 Orange Street, Bloomfield, NJ 07003

Sabatier, Alain M., Rhone-Poulenc Sante, 13 Quai Jules Guesde,,Bl 14F Vitry-Sur-Seine, Cedex, France 94407

1078411

Sanders, Johann, Gist Brocades, P.O. Box 1, Delft, The Netherlands 2600 MA

Sandler, Paula L., University of Wisconsin Medical School, Pharmacology Dept. 1300 University Avenue, Madison, WI 53706

Sarmientos, Paolo, Genetica SA, 160 Quai de Polangis, Joinville-le-Pont, France 94340

Schatzman, Allan, Smith Kline and French Research Laboratories, P.O. Box 7929, Philadelphia, PA 19101

Schauer, Alan T., Harvard University, 99 Hammond Road, Belmont, MA 02178

Schekman, Randy, University of California, Dept. of Biochemistry, Berkeley, CA 94720

Schendel, Paul F., Genetics Institute, 225 Longwood Avenue, Boston, MA 02115

Schoemaker, Joyce M., University of Chicago, Dept. of Microbiology, 920 E. 58th St., Chicago, IL 60637

Schoner, Brigitte E., Lilly Research Laboratories, 307 E. McCarty St., Indianapolis, IN 46285

Schoner, Ronald G., Eli Lilly & Company, Molecular Genetics Dept., 307 E. McCarty Street, Indianapolis, IN 46285

Schottel, Janet L., University of Minnesota, Biochemistry Dept., 1479 Gortner Ave., St. Paul, MN 55108

Schrempf, Hildgund, Dalhousie University, Microbiology, Halifax, Nova Scotia, Canada B3H 4H7

Scott, John, University of Illinois, Department of Microbiology, 131 Burrill Hall, 407 S. Goodwin, Urbana, IL 61801

Sebek, Oldrich K., , 1002 Short Road, Kalamazoo, MI 49008

Seno, Eugene T., Eli Lilly & Co., 307 E. McCarty Street, Indianapolis, IN 46285

Shalita, Zamir P., Rutgers University, Dept. of Biochemistry & Microbiology, P.O. Box 231, Cook College, New Brunswick, NJ 08903

Shaw, Karen J., Schering-Plough, 60 Orange St., Bloomfield, NJ 07003

Shelton, Earl R., Syntex Research, 3401 Hillview Ave., (R1-215), Palo Alto, CA 94304

Sherman, Mary M., University of Wisconsin, School of Pharmacy, 425 N. Charter Street, Madison, WI 53706

Shrote, Jennifer S., Eli Lilly & Co., 307 E. McCarty St., Indianapolis, IN 46285

Shultz, John W., Miles Laboratories, Inc., 1127 Myrtle St. (Bldg. 17), P.O. Box 932, Elkhart, IN 46515

1078412

Silhavy, Thomas, Frederick Cancer Research Center, Cancer Biology Program, P.O. Box B, Frederick, MD 21701

Skatrud, Paul L., Eli Lilly and Company, 307 East McCarty Street, Indianapolis, IN 46285

Smith, Elizabeth B., Schering Corporation, 60 Orange Street, B-9-1, Bloomfield, NJ 07003

Smith, Martin W., The Upjohn Company, Infectious Diseases Research, Kalamazoo, MI 49001

Smith, Robert A., Collaborative Research Inc., 128 Spring St., Lexington, MA 02173

Smolin, David E., American Cyanamid Company, Medical Research Division, Lederle Laboratories, Pearl River, NY 10965

Solenberg, Patricia J., Lilly Research Laboratories, Molecular Genetics Research, 307 E. McCarty St., Indianapolis, IN 46285

Somerville, Ronald L., Purdue University, Dept. of Biochemistry, West Lafayette, IN 47907

Somkuti, George A., Eastern Research Regional Research Center, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118

Speedie, Marilyn K., University of Maryland, School of Pharmacy, UMAB, 20 N. Pine, Baltimore, MD 21201

Srinivasan, V. R., Louisiana State University, Dept. of Microbiology, Baton Rouge, LA 70803

St. John, William D., Biotechniques Laboratories, Inc., 14910 NE 31st Circle, Redmond, WA 98052

Stanzak, Richard K., Lilly Research Laboratories, 307 E. McCarty Street, Indianapolis, IN 46285

Stark, William H., Stark Company, 873 Van Dyke Avenue, Cincinnati, OH 45226

Stassi, Diane L., BioTechnica, International, 85 Bolton Street, Cambridge, MA 02140

Steinberg, Deborah A., Lederle Labs, N. Middletown Road, Pearl River, NY 10965

Stephens, Michael A., Biotechnica International, 85 Bolton Street, Cambridge, MA 02140

Steuerwald, Donna L., The Upjohn Company, 7171 Portage Road, Kalamazoo, MI 49001

Stewart, Graham, Labatt Brewing Company LTD, Box 5050, 150 Simere Street, London, Ontario, Canada N6A 4M3

Streicher, Stanley L., Merck & Co., P.O. Box 2000, Rahway, NJ 07065

Strohl, William R., Ohio State University, Dept. of Microbiology, 484 West 12th Avenue, Columbus, OH 43210

Stroup, Diane, Indiana University, Dept. of Microbiology, 421 South Woodlawn, Bloomington, IN 47401

1078413

Stuttard, Colin, Dalhousie University, Microbiology Dept., Tupper Medical Building, Halifax, Nova Scotia, CANADA B3H 4H7

Sullivan, Mark A., Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35255-5305

Surowitz, Kenneth G., Ohio State University, Dept. of Microbiolgy, 484 W. 12th Avenue, Columbus, OH

Sutcliffe, Joyce A., Abbott Laboratories, Dept. 47M, Bldg. AP9A/Room 218, N. Chicago, Il 60064

Swartling, James R., Eli Lilly & Company, 307 E. McCarty St., Indianapolis, IN

Taylor, Dean P., Smith Kline & French Laboratories, 1500 Spring Garden Street, P.O. Box 7929, Philadelphia, PA 19101

Thill, Gregory, The Salk Institute Biotechnology/Industrial Assn., P.O. Box 85200, San Diego, CA 92138

Tomich, Che-Shen Chiu, The Upjohn Company, Molecular Biology Research, 7242-209-7, Kalamazoo, MI 49001

Tomich, Paul K., The Upjohn Company, Infectious Diseases Research, 7254-25-2, Kalamazoo, MI 49001

Treadway, Patti J., Eli Lilly and Company, 307 E. McCarty Street, Indianapolis, IN 46285

Tuan, James S., Abbott Laboratories, Abbott Park, North Chicago, IL 60064

Turner, Jan R., Eli Lilly & Co., 307 E. McCarty St., Indianapolis, IN 46032

Uchiyama, Hiroo, University of Wisconsin Medical School, Pharmacology Dept., 1300 University Avenue, Madison, WI 53706

Ueng, Pear P., Purdue University, 154 Prophet Drive, W. Lafayette, IN 47906

Veal, Loraine E., Lilly Research Laboratories, 307 E. McCarty Street, Indianapolis, IN 46285

Volpp, Kristi J., Purdue, 514 Evergreen St., W. Lafayette, IN 47906

Vournakis, John, Syracuse University, Dept. of Biology, Biological Research Laboratories, 130 College Place, Syracuse, NY 13210-2878

Wall, Judy D., University of Missouri-Columbia, Biochemistry Dept., 322 Chemistry Bldg. UMC, Columbia, MO 65211

Walrud, Lidia S., Monsanto, 800 N. Lindbergh, St. Louis, MO 63167

Warren, Thomas G., Unigene Laboratories Inc., 110 Little Falls Road, Fairfield, NJ 07006

1078414

Weber, J. Mark, Harvard University, Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138

Weeks, Robert E., Eli Lilly and Company, 307 E. McCarty St., Indianapolis, IN 46285

Weisblum, Bernard, University of Wisconsin Medical School, Pharmacology Dept., Madison, WI 53706

Westpheling, Janet, Harvard University, The Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138

Wickner, William, University of California, Dept. of Biological Chemistry, School of Medicine, 405 Hilgard Avenue, Los Angeles, CA 90024

Wilson, C. Ron, Dow Chemical Co., CR-Biotechnology Lab, 1701 Bldg, Midland, MI 48640

Wollweber, Karen L., Miles Laboratories, Inc., 1127 Myrtle St. (Bldg. 17), P.O. Box 932, Elkhart, IN 46515

Wolnak, Bernard, Bernard Wolnak & Associates, 360 N. Michigan Avenue, Suite 706, Chicago, IL 60601

Womble, David D., Northwestern University, Dept. of Molecular Biology, The Medical School 303 E. Chicago Avenue, Chicago, IL 60611

Wood, John S., Eli Lilly and Co., 307 E. McCarty Street, Indianapolis, IN 46285

Wovcha, Merle G., The Upjohn Co., 7171 Portage Road, Kalamazoo, MI 49002

Wu, Tzay-Shiang, Ohio State University, Dept. of Chemistry, 140 W. 18th Ave., Columbus, OH 43210

Wulf, John J., Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94080

Yablonsky, Michael D., Rutgers University/Cook College, Lipman Hall, Biochemistry and Microbiology, New Brunswick, NJ 08903

Yagi, Yoshihiko, The Upjohn Company, Unit 7254, Kalamazoo, MI 49001

Yamamoto, Hirokazu, University of Wisconsin, 425 N. Charter St., Madison, WI 53706

Yasbin, Ronald E., University of Rochester Medical Center, Dept. of Microbiology, Box 672, 601 Elmwood Avenue, Rochester, NY 14642

Yeh, Wu-Kuang, Eli Lilly & Co., 307 E. McCarty St., Indianapolis, IN 46285

Zabeau, Marc, Plant Genetics Systems NV., Free University Brussels, J. Plateaustraat 22, Gent, Belgium 9000

Zmijewski, Jr., Milton J., Eli Lilly and Co., 307 E. McCarty St., Indianapolis, IN 46032

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ASM CONFERENCE ON GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL MICROORGANISMS

ON SITE REGISTRATION

SEPTEMBER 30, 1984

Bittner, Michael, Monsanto, 800 N. Lindbergh, St. Louis, MO 63167

Bolen, Paul, USDA, Northern Regional Laboratories, 1815 N. University St., Peoria, IL 61604

Burnett, William, Bristol-Myers, Genetic Engineering Dept., P.O. Box 4755, Syracuse, NY

Deo, Yashwant, Schering Corporation, Biotechnology Development, 1011 Morris Ave., Union, NJ 07083

Firpo, Eduardo, Instituto Cientifico Paul, CC 39-1708 Moron, Buenos Aires, Argentina

Gargiulo, Vickie, Lederle Laboratories, Biotechnology Research, Middletown Road, Pearl River, NJ 10965

Ginsburgh, Charles, CPC International, Dept. of Biotechnology, MOffett Technical Center, Summitt-Argo, IL 60501

Gramdi, Guido, Assoremi, Molecular Biology Dept., Fabiani, Milan, Italy 20097

Kaplan, Lou, Merck & Co., Inc., 7 Lexington Rd., Rahway, NJ 07065

LaTorre, Jose Leonard, Cevan, Serrano 661, Buenos Aires, Argentina

Loper, John C., University of Cincinnati, Microbiology and Molecular Genetics, Cincinnati, OH 45267

MacKellar, Warren, Eli Lilly and Co., 307 E. McCarty St., Indianapolis, IL 46217

Mahro, Bernard, Massachusetts Institute of Technology, Cambridge, MA 02139

McCarthy, Mary Ellen, McGraw-Hill Biotechnology Newswatch, 1221 Avenue of the Americas, New York, NJ 10020

Proctor, Alan, Pfizer Central Research, Molecular Genetics, 17 Mohegan Road, Noank, CT 06240

Rennick, Kathy, USDA-NRRC, 1815 N. University, Peoria, IL 61604

Rosen, Ira, Kelco Division, Merck & Co., Inc. Genetics & Physiology Dept., 8225 Aero Drive, San Diego, CA 92123

Shepherd, Hurley, USDA, Northern Regional Laboratories, Peoria, IL 61604

Wright, Fred, Pfizer Central Research, Molecular Genetics Dept., Eastern Point Road, Groton, CT 06340

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SEPT. 30, 1984 (cont.)

Zamost, Bruce, Novo Laboratories, 59 Danburg Road, Wilton, CT 06897

OCTOBER 1, 1984

Dackowski, Bill, Integrated Genetics, Yeast Expression, 31 New York Avenue, Framingham, MA 01701.

Elliott, Diane, Eli Lilly & Co., Fermentation Tech., 307 East McCarthy Street, Indianapolis, IN 46228.

Grindley, June, Biogen, 14 Cambridge Centre, Cambridge, MA 02142.

Ho, Nancy, Purdue University, Lorre, West Lafayette, IN 47906.

Oh, Yong, Smithkline-French Labs, Philadelphia, PA 19101.

Laskin, Allen, Exxon Research and Engineering, Biosciences, Route 22 East, Annandale, NJ 08801.

Meade, Harry, Biogen, 14 Cambridge Centre, Cambridge, MA 02142.

*Raghunathan, R., Meharry Medical College, Obst. & Gynecol. Div. Res., 1005 18th Avenue, North, Nashville, TN 37208.

Slepecky, Ralph, Syracuse University, Biology, 130 College Pl., Syracuse, NY 13210.

Stevis, Panayiotis, Purdue University, Lorre, West Lafayette, IN 47906.

Zavela, David, Eli Lilly & Co., Fermentation Tech., 307 East McCarthy Street, Indianapolis, IN 46228.

VOLUNTEERS FROM INDIANA UNIVERSITY

Cheryl Blake
Tom Burkey
Jerry Cane
Joe Dolan
Randy Elble
Dr. Brian Fry
Heidi Giordano
Karen Harris
Uwe Heine
Prof. Arthur Koch
Prof. Walt Konetzka
Fred Quinn
Sabine Rundle
Mike Schmidt
Gabriella Vasquez
Scott Watkins
Prof. David White
Steven Woeste
Ed Wong
Erin Zucker

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ASM Conference Report: Genetics and Molecular Biology of Industrial Microorganisms 1984

The ASM Conference on Genetics and Molecular Biology of Industrial Microorganisms (GMBIM) was held 30 September to 3 October 1984. The proceedings will be published in Microbiology—1985 *by the American Society for Microbiology, Washington, D.C.*

The campus of Indiana University in Bloomington was the scene of the third conference in a series sponsored by ASM and held every 4 years since 1976. The conference last met in Bloomington in 1980 (3) and has alternated biennially with the international Genetics of Industrial Microorganisms (GIM) meetings, the most recent of which was held in Kyoto, Japan, in 1982 (1, 4). This meeting has become an excellent forum for bringing together scientists from the industrial and academic sectors. It affords an opportunity to discuss the genetics and molecular biology of organisms that are of interest because of their industrial applications and their contributions to our understanding of basic biological processes. In addition to the traditional interests, which include the antibiotic-producing actinomycetes and fungi, the list of industrial microorganisms has expanded to include *Escherichia coli* and other organisms finding applications as a result of the impact of recombinant DNA technology. The meeting's format provided a sampling of the work being done in various areas relating to genetics, molecular biology, gene expression, and protein transport in these industrial microorganisms.

The meeting itself had three sessions per day, with an additional hour or two set aside for poster sessions on each of the two full days. This schedule did not permit much free time to see Bloomington or the campus, to enjoy the splendid fall weather, or to recapture one's youth. The time available for discussion after the talks was generally adequate, and the mixers and meals after each session provided excellent opportunities for continued discussion. The people from Eli Lilly & Co. continued their practice of providing single-page reductions of their posters, a practice that was widely appreciated. The tenor of the meeting favored molecular biology more than the classic approaches, reflecting the progress in applying molecular techniques to the genetic analysis of industrial microorganisms.

Some basic themes were evident which distinguished this meeting from earlier meetings. Much of the promise held forth at GMBIM 1980 and GIM 1982 is now being realized, and products generated by research, particularly those resulting from recombinant techniques, are now on the market or in development. Our degree of understanding at the molecular level has expanded considerably, and not surprisingly, some variations are appearing. For example, the finding of multiple promoters or upstream regulatory elements is more prevalent. The complexity of some of the systems is now better appreciated, and that knowledge is having considerable impact on applications, including heterologous gene expression, overproduction of proteins, and antibiotic improvement. In the last several years, the application of recombinant DNA technology to *Streptomyces* species has made it possible to clone, study the organization of, and even manipulate antibiotic biosynthesis pathways. Cloning in the yeast *Saccharomyces cerevisiae* has become an accepted practice, but such work in fungi has been possible only in the last few years. Now molecular cloning appears to be feasible even in antibiotic-producing fungi such as *Cephalosporium* and *Penicillium*.

The meeting opened with a welcome by G. Hegeman of Indiana University, followed by three plenary lectures. Julian Davies (Biogen, Geneva, Switzerland) reviewed the use of recombinant DNA techniques to modify the production of small molecules, including primary metabolites, antibiotics, alkaloids, and other secondary metabolites. David Jackson (Genex Corp.) discussed the advent of protein engineering by recombinant DNA methodology but also stressed the importance of knowing the structure of proteins and highlighted some recent advances which allow rapid elucidation of protein structure. Graham Stewart (Labatt Brewing Co.) described the considerations, difficulties, and some successes in genetically manipulating the yeast strains employed in brewing. He made an interesting point regarding the necessity of introducing a mutation (*pof*) required to eliminate the production of off-flavors by strains used to produce light beer, demonstrating to some in the audience the impact that such work can have on our daily lives.

To review some of the specific results presented at the conference, I have grouped the talks on a taxonomic basis. In considering gene expression in yeasts, L. Guarante (Massachusetts Institute of Technology) identified the promoter and regulatory elements of the *cyc1* gene of *S. cerevisiae* by using *lacZ* fusions. As has been observed in other systems, the promoter requires a TATA sequence at −30 base pairs (bp) upstream from the transcription start site, but interestingly, another *cis*-acting element, an upstream activation sequence (UAS), is required. The *cyc1* gene has two of these sequences, UAS1 at −275 bp and UAS2 at −225 bp, which are required for heme regulation and catabolite repression of *cyc1* expression. J. Meade (Cetus Corp., Emeryville, Calif.) described the cloning of an *Aspergillus* sp. gene for glucoamylase into *S. cerevisiae*. Despite sequence similarities, the *Aspergillus* promoter was not recognized and the introns in the *Aspergillus* glycoamylase gene were not properly excised by the yeast strain. The glucoamylase gene was then engineered to delete the four introns and placed behind the *ENO1* promoter, permitting glucoamylase production, glycosylation, and transport to take place. Also in *Aspergillus*, the developmental regulation of gene expression of the sporulation gene cluster (*spoC1*) was followed through conidiophore development by examining the appearance of polyadenylic acid-containing transcripts (B. Miller, University of Cali-

fornia–Davis). One level at which these transcripts may be regulated is the level of chromatin structure. This regulation is dependent on chromosome location but is not disrupted by insertion into the *spoC1* cluster. John Scott (University of Illinois, Urbana) described the construction of a yeast acentric ring plasmid (YARp1) and found that it is in a nucleosomelike structure in *S. cerevisiae* and that it replicates similarly to the original chromosomal segment although at a copy number of 100 to 200. Cloning vectors derived from YARp1 and their utility were described. Extending the range of recombinant DNA technology to *Cephalosporium*, the successful cloning of hygromycin resistance genes into this organism was reported by S. Queener (Eli Lilly & Co.). The vector used to transform *Cephalosporium* contained an autonomously replicating sequence isolated from yeast, but the transformed DNA appeared to have integrated into the *Cephalosporium* chromosome rather than remaining autonomous. Although the transformation frequency (1 transformant per 10 μg of DNA) will need to be increased, at least it's a beginning.

The newest industrial microbe, *E. coli*, clearly owes its prominence to our extensive knowledge of its genetics. A. Shatzman (SmithKline, Philadelphia) described work done with *E. coli* to optimize gene expression and argued convincingly that a system optimized for expression of a particular gene may not be optimized for the expression of other genes. Larry Gold (University of Colorado, Boulder) presented a model for the initiation of translation in *E. coli* based on experiments with T4rIIb mutants. He also offered an explanation for the efficiency of expression of translation initiation factor 3 (IF-3) by describing the structural changes that may be occurring in the ribosome to initiate translation of IF-3 mRNA independently of IF-3. A. Goldberg (Harvard University) described the role of proteases in protein turnover, going into detail on the ATP-requiring protease produced by the *lon* gene, but examples of the effect this protease might have on the accumulation of heterologous proteins by *E. coli* were not given. Control of replication of the *E. coli* plasmid ColE1, the prototype for the popular pBR322 cloning vector, is mediated by the interaction of a small RNA with another RNA which serves as the primer for the initiation of DNA synthesis. B. Polisky (Indiana University) presented experimental evidence indicating which segments on these RNAs are needed for this interaction. Methods for probing the secondary structure of RNA, including the use of methidium-propyl EDTA-Fe(II), were aptly demonstrated by J. Vournakis (Syracuse University). The application of various gene expression systems for the production of vaccines was described by D. Kleid (Genentech) with the notable inclusion of the use of mammalian cells as gene expression systems for antigen production.

Gene expression in *Bacillus* species was represented as well and is particularly interesting as a system clearly being developed on its own merits but which bears watching because of its similarities or analogies to *Streptomyces*. R. Losick (Harvard University) has been examining the sporulation genes of *Bacillus*, particularly the *spo5G* gene, as a model of developmentally regulated genes. The promoter for *spo5G* was fused to *lacZ* on phage SPβ for introduction into various mutant hosts, demonstrating that three *spoO* genes are required for induction. The *spo5G* promoter region contains two sets of −10 and −30 regions and two transcription start sites plus an AT box upstream that enhances promoter function. To facilitate analysis of gene expression in *Bacillus* generally, a derivative of transposon Tn*917* was described which contains the *E. coli lacZ* gene with a *Bacillus* ribosome binding site appended. This transposon is carried on a temperature-sensitive plasmid so it can insert readily into the chromosome; such events can be selected for by antibiotic resistance. Interrupted genes can be studied by fusing the promoter upstream of the insertion site to the *lacZ* gene on the transposon. Developmentally regulated genes can be detected by the regulated expression of *lacZ* with 4-methylumbelliferyl-β-D-galactoside, a fluorogenic substrate for β-galactosidase. Unfortunately, this transposon does not appear to work in *Streptomyces* species. P. Lovett (University of Maryland) discussed the inducible chloramphenicol resistance found in *Bacillus* species; a stem-loop structure may play a role in the regulation of expression of this gene. Interestingly, screening chloramphenicol-related antibiotics led to finding a chloramphenicol analog, amicetin, which will induce expression.

The work presented on *Streptomyces* systems was impressive both in its volume and in its contribution to our understanding of this organism. The topics described included gene expression, antibiotic production, DNA rearrangements, plasmids, phages, and insertion elements. The examination of gene expression in *Streptomyces* by using various gene fusion systems to examine promoters was described. Using *E. coli* genes for kanamycin or chloramphenicol resistance, G. Janssen (John Innes Institute, Norwich) compared various promoters derived from *Streptomyces* antibiotic resistance determinants. Interestingly, two transcription start sites were identified for some of the promoters analyzed. Comparison of the sequences failed to reveal a consensus promoter sequence, and comparisons with *E. coli* and *Bacillus* promoters showed incomplete homology but no convincing demonstration of their role in promoting transcription. Of particular interest was a report by J. Fayerman (Lilly) that the *veg* promoter of *Bacillus* can express *E. coli* β-galactosidase and hygromycin phosphotransferase activities in *Streptomyces ambofaciens*. This promoter is also recognized in vitro by one form of *Streptomyces* RNA polymerase (J. Westpheling, Harvard University). The *Streptomyces* β-galactosidase gene region was characterized, and three promoters contained in that region were reported by T. Eckhardt (SmithKline), as was the expression of *E. coli* galactokinase transcribed by one of these promoters in *Streptomyces* (M. Brawner, SmithKline). Even the P_L promoter of coliphage

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lambda was reported to express the phosphotransferase of Tn*5* in *Streptomyces* (N. Rao, Lilly).

The cloning of genes involved in antibiotic biosynthesis was described by D. Hopwood (John Innes Institute). In addition to reviewing the strategies that he and his collaborators have used recently to clone antibiotic biosynthesis genes, including the entire pathway for actinorhodin (5), he presented some interesting results obtained by using cloned actinorhodin genes. Working with S. Omura and colleagues (Kitasato Institute, Tokyo), they found that when a plasmid carrying genes for part of the actinorhodin pathway was introduced into a producer of medemycin, another isochromanequinone antibiotic related to actinorhodin, the organism produced a hybrid antibiotic. The results were colorful because the hybrid antibiotic, mede-rhodin, is a pigmented antibiotic clearly distinct from both parent compounds. However, when the entire pathway for actinorhodin was introduced, only the two individual antibiotics were produced. One of the strategies for cloning in *Streptomyces* species, mutational cloning (2), as applied to primary metabolism genes and to the genes for production of methylenomycin was described by K. Chater (John Innes Institute). This procedure relies on cloned DNA to provide the homology for integration of the phage genome into the chromosome since the attachment site has been deleted from the phage. When the cloned DNA is derived from the methylenomycin region, genes for its production can be interrupted. One "mutational clone" gave increased production of methylenomycin, indicating that a negative control may have been inactivated. Analysis of other mutational clones has indicated that the production genes are clustered on either side of the resistance gene, a fortuitous arrangement for gene cloners if it applies to other antibiotic production genes. Another method to improve antibiotic production was described by B. Weisblum (University of Wisconsin–Madison), who demonstrated that maridamycin selection of *Streptomyces lincolnensis* mutants constitutive for resistance produced fivefold more lincomycin. The cloning of the gene involved in actinomycin D biosynthesis was presented by G. Jones (University of Michigan–Ann Arbor). The gene for phenoxazinone synthetase from *Streptomyces antibioticus* was cloned in *Streptomyces lividans* and detected by using a sensitive enzyme assay. The surprise came when different fragments were identified in three of the synthetase producers. One of these fragments was shown to code for the synthetase, and the other two are thought to encode functions which induce a silent *S. lividans* gene to express itself; this work will be interesting to watch as it is explored further.

Streptomyces species have long been noted for unstable phenotypes and variable colonial morphology, implying genetic instability. Now, the basis of that genetic instability is being investigated at the DNA level with interesting results. H. Shrempf (Dalhousie University) has seen deletions of the tyrosinase gene in *S. antibioticus* associated with DNA amplification and rearrangements; some of the sequences involved occur in most *Streptomyces* strains. Similar observations were made for the *Streptomyces glaucescens* tyrosinase gene; but in addition, G. Hintermann (Zurich) presented evidence that high-frequency loss of streptomycin resistance and production is due to large deletions. The structure of an amplified DNA segment was characterized by C. Hershberger (Lilly). The amplified DNA contains head-to-tail repeats generated by reiteration of a 10.5-kilobase (kb) segment bounded by 2.2 kb of directly repeated sequences. With these regions as probes, other related sequences and their organization are being studied.

Other genetic elements would be expected to play a role in DNA rearrangement, namely insertion elements, and again *Streptomyces* has not disappointed us. Chater et al. reported a 1.6-kb segment of DNA

Upcoming ASM Conferences

Virus Attachment and Entry Into Cells

The ASM Conference on Virus Attachment and Entry Into Cells will be held Wednesday through Saturday, 10–13 April 1985, at the Franklin Plaza Hotel in Philadelphia, Pa. The preliminary program and preregistration and housing forms for this meeting appeared in the February 1985 issue of *ASM News*. Further information is available by writing to the ASM Conferences Department at the address below.

Molecular Aspects of Protein Secretion and Membrane Assembly: Deadline for Abstracts

The ASM Conference on Molecular Aspects of Protein Secretion and Membrane Assembly will be held Sunday through Wednesday, 12–15 May 1985, at the South Bend Marriott Hotel in South Bend, Ind. Contributed papers will be presented in the poster sessions. Those wishing to contribute should submit an abstract on an official abstract form no later than 18 March to the ASM Conferences Department at the address given below. Official abstract forms can be obtained by contacting the ASM Conferences Department. Attendance will be limited to 300 participants. The preliminary program and preregistration and housing forms for this meeting appeared in the January 1985 issue of *ASM News*.

For further information on these upcoming conferences, contact the Conferences Department, American Society for Microbiology, 1913 I St. NW, Washington, DC 20006, (202) 833-9680.

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which was acquired by an *att*-deleted φC31 phage, permitting the phage to integrate into the *Streptomyces coelicolor* chromosome at several sites. This sequence, designated IS*110*, is present in several copies on the genome of *S. coelicolor* but absent in *S. lividans*. D. Lydiate (John Innes Institute) observed a 2.6-kb sequence which could exist as covalently closed circular DNA with a copy number of 0.2 and demonstrated that it exists as an integrated sequence as well, suggesting that this DNA may also move around the genome like a transposon. Another well-known DNA sequence of *S. coelicolor*, SLP1, can exist as a plasmid or integrated into the chromosome in *S. lividans*. Evidence for a mechanism based on site-specific excision and integration of SLP1 has come from studies by C. Omer (Stanford University) which show some similarities to bacteriophage lambda (6). A *Streptomyces fradiae* phage was also described which, like the *E. coli* phage P1, can exist as a plasmid but propagate as a phage (S.-T. Chung, Upjohn Co.). The meeting was replete with reports on new plasmids, phages, and vectors, which will require additional studies to ascertain their importance.

The topic of protein secretion in three systems was addressed by four speakers. The pathway used by *S. cerevisiae* to transport invertase to the Golgi body was reviewed by R. Shekman (University of California–Berkeley), who described the effects that glycosylation and various mutations have on transport. J. O. Lampen (Rutgers University) compared the signal sequences of *Bacillus* proteins and the typical sequences of gram-negative organisms. Using the M13 coat protein to study translocation of protein into the *E. coli* membrane, W. Wickner (University of California–Los Angeles) described some clever experiments that allowed overproduction of the peptidase that converts M13 procoat to coat protein and to follow cleavage and insertion of the protein into the membrane. L. Randall (Washington State University, Pullman) explained that the secretion of proteins into the periplasm of *E. coli* is not coupled to polypeptide elongation but requires proton motive force for translocation. Again it is obvious that the better-studied systems have the advantage, but it will only be a matter of time before more is known about the secretion of proteins by other organisms. The reports of exocellular proteins produced by *Streptomyces* species included β-galactosidase, tyrosinase, agarase, and esterase.

As is apparent, the topics covered by the meeting were diverse, reflecting the attendance which could be arbitrarily broken down as follows: 35% academic, 35% industrial, 14% biotechnological, and 16% other various groups. The organizers, Dr.'s Hershberger and Hegeman and colleagues, are to be commended for organizing a meeting which can bring together such a diverse group and seemingly please the majority. Moreover, they are to be respected for their willingness to repeat the task of organizing the next meeting, to be held in Bloomington in 1988. Given the rate of progress, we can expect to hear about applications of cloning to commercially interesting antibiotics, protein export, and production of proteins of pharmaceutical importance, as well as areas that the next 4 years will determine to be relevant.

Dean Taylor
Dept. of Molecular Genetics
SmithKline & French Laboratories
Philadelphia, PA 19101

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Publications

MCB Annual Report

Molecular and Cellular Biology (MCB) this past year has again grown in quantity and quality. A total of 660 manuscripts were processed, compared with 500 in 1983 (Fig. 1). This represents an increase of more than 30%. Volume 4, published in 1984, consisted of 389 original reports and 2,936 pages.

The rapid rise of MCB to a position of importance among scientific publications has been possible because of the genuine interest and active participation of many. More than 300 ad hoc reviewers, listed in the December issue, provided invaluable advice to the editors and editorial board members. On-schedule publication was also facilitated by the committed efforts of ASM Publications Board Chairman Helen R. Whiteley, Director of Publications Walter G. Peter III, Managing Editor, Journals, Linda M. Illig, and the many others in the ASM Publications Office.

The scope and goal of MCB—to advance and disseminate fundamental knowledge concerning the molecular biology of eucaryotic cells of both microbial and higher orga-

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ABSTRACTS

OF THE

ASM CONFERENCE ON GENETICS AND MOLECULAR BIOLOGY

OF INDUSTRIAL MICROORGANISMS

30 SEPTEMBER - 3 OCTOBER, 1984

UNIVERSITY OF INDIANA MEMORIAL UNION

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101 High level, inducible resistance to tetracycline encoded on the chromosome of *Bacillus subtilis*

Alice Morgan and Ron Wilson
The Dow Chemical Company
Central Research
Biotechnology Laboratory
1701 Building
Midland MI, 48640

In the process of transforming *Bacillus subtilis* BC92 with a chloramphenicol (cm) and tetracycline (TC) resistance plasmid (pCW59) from *Staphylococcus aureus*, a few $Cm^S Tc^R$ transformants were isolated. All of the transformants exhibited high level inducible resistance to tetracycline. However, no plasmid DNA was detected in any of the transformants either by density gradient centrifugation or agarose gel electrophoresis. Isolation of $Cm^S Tc^R$ transformants did not occur in the absence of pCW59 DNA. Phage PBS1 was used to map Tc resistance on the *B. subtilis* chromosome. The possibility for the integration of the pCW59 Tc^R determinant into the *B. subtilis* chromosome is discussed.

102 CONSTRUCTION OF VERSATILE GENE FUSION VECTORS FOR *BACILLUS SUBTILIS*.

Mark A. Sullivan, Microbiology Division, Southern Research Institute, Birmingham, AL

Two plasmid vectors have been constructed which allow efficient isolation and characterization of *lacZ* gene fusions in *B. subtilis*. Both vectors replicate and confer ampicillin resistance in *E. coli* and have an intact *lacZ* gene containing an array of unique restriction sites in the N-terminal portion of the gene. Insertion at these sites of *B. subtilis* DNA fragments which contain a promoter and an N-terminal protein sequence in frame with the remainder of *lacZ* will yield a Lac^+ phenotype in *B. subtilis*. One vector, pMAS100, confers chloramphenicol resistance in *B. subtilis*, but lacks an origin of replication. Cm^r transformants arise by integration via homology with the inserted promoter, resulting in only one copy of the fusion per cell. The other vector, pMAS101, confers kanamycin resistance and replicates as a plasmid in *B. subtilis* yielding 20-40 copies of the fusion per cell.

To demonstrate the utility of these plasmids, a DNA fragment containing the regulatory region and eight amino acids of the leader sequence of the extracellular serine protease of B. subtilis (*sprE*) has been cloned in both plasmids. In the integrated form, β-galactosidase is synthesized only at the end of exponential growth in sporulation medium. In medium which represses sporulation due to a high glucose concentration, post-exponential induction of β-galactosidase is not observed. When the fusion is present on a multicopy plasmid, elevated levels of β-galactosidase are present during exponential growth, but a 3- to 4-fold induction is still observed as the cells enter stationary phase.

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103 Secretion of Staphylococcal Nuclease in Bacillus subtilis.

S. KOVACEVIC, L. E. VEAL, H. M. HSIUNG and J. R. MILLER, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

The staphylococcal nuclease (nuc) gene from Staphylococcus aureus has been cloned and expressed in Bacillus subtilis. The nuclease protein was expressed either from its own promoter and translation start signals or from a combination of a Bacillus promoter, ribosome binding site, and signal peptide sequence. Greater than 80 percent of active gene product was secreted into the medium, whereas, when a signal peptide sequence was absent, only 10 percent of the nuclease activity was found in the culture medium. Intracellular (or cell-bound) nuclease, as determined by Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis and Western blotting, was shown to have the molecular weight predicted by the length of the signal peptide. Levels of nuclease reached 50 mg per L in the culture medium depending on the growth medium and strain used. These findings indicate the prospective use of nuclease as a model system for studying secretion of heterologous proteins in B. subtilis.

104 Construction and use of a lacZ fusion vector for Bacillus.

P.M. ANDREOLI, H.N. SCHEFFERS, C.H.C. VAN VELDHOVEN and J.P.M. SANDERS*, Dept. of Bacterial Genetics; Gist-Brocades N.V., Delft - The Netherlands.

The β-galactosidase structural gene lacZ can be fused to the promotor and controlling elements of other genes, as a way to provide an enzymatic assay for gene expression. In order to apply such a system in Bacillus, we have constructed the lacZ vector pGBG3415. This dual-host vector was constructed by the E.coli plasmid pMLB1034 (which has unique EcoRI, SmaI and BamHI sites within the first 24 bp of the lacZ gene) and the Bacillus plasmid pBD15 (which allows replication and provides for erythromycin resistance (Em^r) in B.subtilis). To show that pGBG3415 can be used to isolate and identify restriction fragments with promotor activity, we digested chromosomal B.licheniformis DNA with EcoRI* or Sau3A and ligated the fragments to EcoRI or BamHI digested pGBG3415. Among the Em^r E.coli-LG90 transformants obtained with this ligated DNA only 4% formed red colonies on MacConkey indicator plates. Transformation of B.subtilis with plasmid DNA isolated from these red colonies, yielded pale blue to dark blue Bacillus transformants on Spizizen agar plates containing Xgal. The properties of these promotor lacZ fusion plasmids will be presented with special emphasis on their promotor strength, and their stability in E.coli as well as in B.subtilis

105 THE MLS RESISTANCE MESSENGER RNA OF PLASMID PE194:

DEMONSTRATION OF ITS CONFORMATIONS AND OF THE LEADER PEPTIDE THAT IT ENCODES

Mark Mayford and Bernard Weisblum
University of Wisconsin, Madison, WI

Co-resistance to 3 chemically distinct groups of antibiotics, namely, the macrolides, lincosamides, and streptogramin B (MLS) antibiotics comprises a resistance syndrome of bacteria in which biochemical alteration of the ribosome, namely N6-methylation of a single adenine residue in 23S rRNA confers resistance to all members belonging to the three groups. Both inducible and constitutive forms of MLS resistance are found in strains isolated from nature. One inducible form of MLS resistance specified by a small Staphylococcus aureus plasmid, pE194, has provided a model system in which to study the biochemical basis of induced MLS resistance. Specific active and inactive conformations of the MLS methylase mRNA comprise key features of the attenuation model according to which the mRNA assumes an active conformation as a consequence of internal rearrangement. This rearrangement, in turn, is causally linked to inhibition by an inducing antibiotic, of the synthesis of a hypothetical 19 amino acid peptide whose corresponding structural gene overlaps part of the control region. It is postulated that the conformational shift which occurs unmasks the ribosome loading site sequestered by the inactive conformation that the mRNA assumes during growth under non-inducing conditions. Strong support for the model of induction has come from genetic studies involving constitutive mutants in which expression of MLS resistance was correlated with destabilization of the secondary structure of the MLS mRNA leader sequence.

We have analyzed the conformation of the MLS mRNA more directly using biochemical means in which we cleave gamma-^{32}P-end-labeled transcripts with nuclease probes that digest RNA differentially according to the conformation which the latter assumes. In the present studies the +1 site for transcription initiation of the inducible 23S rRNA adenine methylase encoded by plasmid pE194 was determined experimentally by S1 mapping of mRNA synthesized in vivo, and by T1 mapping of gamma-^{32}P-end-labeled transcripts synthesized in vitro. By partial digestion of the in vitro transcripts using S1-, T1-, and cobra venom nucleases as probes of mRNA conformation, single- and double-stranded regions, respectively, were identified which correspond to the critical stems and loops postulated for active and inactive conformations of the nascent mRNA. According to the model for induction, the transition from inactive to active conformation involves disruption of mRNA secondary structure and this, in turn, occurs as a consequence of protracted occupancy by ribosomes complexed with erythromycin, of one of the critical stem sequences that additionally contains an open reading frame corresponding to part of the 19 amino acid control peptide. The existence of the control peptide, predicted by the nucleotide sequence of the control region, was demonstrated as part of a translational fusion with Escherichia coli beta-galactosidase. The DNA sequence at the amino end of the fusion protein structural gene corresponds to the amino acid sequence MGIFSGDPVVL in which MGIFS- is derived from the pE194 MLS leader control peptide and -GDPVVL is derived from beta-galactosidase. Direct amino acid sequence determination to the extent of 10 residues from the amino end gave GIFSGDPVVL as the N-terminal sequence of the fusion protein.

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106 Isolation of Plasmids Containing B. subtilis 168 DNA that complement the LeuB Gene of E. coli HB101, J. Greenwood; J. Shultz, Miles Laboratories, Elkhart, IN

Two plasmids that allow E. coli strain HB101 leu B, pro, B1) to grow in minimal media lacking leucine were selected from a pool of clones containing Eco R1 Fragments of B. subtilis 168 DNA inserted into the Eco R1 site of pBR322. These two clones, pleu1 and pleu2, were both shown to contain two Eco R1 fragments of B. subtilis DNA of 3.0 and 1.9 kb and 2.4 and 1.9 kb, respectively. The common 1.9 kb DNA fragment was found to be identical in the two clones and was subsequently shown to contain all the DNA needed to complement leuB in E. coli HB101. Southern blot analysis of these clones suggest that they are both clones of the same region of the chromosome - differing only in the flanking DNA segment present with the common 1.9 kb Eco R1 fragment. Neither clone was able to complement B. subtilis strains for either leuA, leuB or leuC. In vivo transcription and translation of the DNA of various clones in E. coli revealed that a 39,500 dalton protein is associated with the ability of these clones to complement leuB.

107 CHARACTERIZATION OF PLASMID DNA FROM THIOBACILLUS FERROOXIDANS BY RESTRICTION ENZYME MAPPING. K. Mattern Dolan* and Olli H. Tuovinen, Department of Microbiology, Ohio State University, Columbus, Ohio 43210.

Thiobacillus ferrooxidans is an acidophilic chemolithoautotroph that has been shown to enhance the solubilization of metals from ore materials. Because of its physiological characteristics, the bacterium shows promise in biohydrometallurgical processess. In the present work, plasmid DNA in previously described strains of T. ferrooxidans TFI-29 and TFI-35 was characterized by cesium chloride-ethidium bromide density centrifugation and restriction mapping. TFI-29 was shown to contain two plasmids, pTF-291 and pTF-292 while only one plasmid, pTF-350 was detectable in TFI-35. Plasmids pTF-291 and pTF-350 were digested separately with six different restriction enzymes: HindIII, PstI, EcoRI, BamHI, HaeIII, and BglI. The plasmids contained a single restriction site for HindIII. PstI digested the plasmids to yield three restriction fragments as did EcoRI, whereas digestion with BamHI produced four linear fragments. Separate digestions with HaeIII and BglI produced numerous small fragments. The positions of the different cleavage sites for HindIII, BamHI, and PstI were determined by digesting the plasmid DNA with appropriate pairs of enzymes. Based on the restriction enzyme data, pTF-291 and pTF-350 are assumed to be the same plasmid. Resistance to Hg^{2+} was also investigated because mercuric reductase, known to be exclusively plasmid mediated, was present in TFI-29. T. ferrooxidans TFI-29 showed a higher level of resistance to mercuric ion than did TFI-35 (10 μM and 5 μM, respectively). The presence or absence of mercuric ion in the growth medium did not affect the plasmid profile of either strain. DNA:DNA homology studies are in progress to locate the mercuric reductase genes.

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GLYCEMIC STATUS OF SIDS SIBLINGS: LEVELS OF THE ENZYME PHOSPHOENOL-PYRUVATE CARBOXYKINASE IN AMNIOTIC FLUID OF SIDS-SIBLINGS.

Rengachari Raghunathan[1], Henry W.Foster Jr.[1], Srinivas.N[2], Donald B.Barnes[3] & Lana Beavers[3]. 1. Division of Research, Department of Obstetrics & Gynecology, Meharry Medical College, Nashville, Tn, 37208; 2. Private Obstetrics Practice, Hendersonville, Tenn, 37075 & 3. Bedford County General Hospital, Shelbyville, Tenn, 37160.

There is considerable controversy regarding a genetic component for Sudden Infant Death Syndrome(SIDS). Among the several theories on the cause of this disease, it has been suggested that in SIDS victims, gluconeogenesis may be impaired in the liver, due to the complete absence of/and or a decreased level of the enzyme, phosphoenolpyruvate carboxykinase(PEPCK). If this hypothesis is true, it would be worthwhile to explore it further, since PEPCK is a key enzyme in the pathway of glucose synthesis and its absence or low levels could lead to very low energy levels in the brain of infants, making the brain to either abruptly stop working or stop transmitting signals to the vital organ, the Heart.

We have recently shown a variation in the levels of the enzyme, PEPCK during gestation in amniotic fluid samples obtained from normal pregnancies. The specific activity of this enzyme in amniotic fluid of normal pregnancies ranged from 5.5. to 14.22 n moles/min/mg protein, depending on the stage of gestation. As opposed to these normal levels, in a SIDS-sibling pregnancy at term, PEPCK activity was found to be 6.2 n moles/ min/mg protein. Comparative levels of PEPCK in amniotic fluid of normal pregnancies obtained at term as shown in our earlier studies was 10.85 n moles/min/mg protein. In another Crib death sibling pregnancy at term, the PEPCK level was found to be 7.4 n moles/min/mg protein. Thus, there is a 32 and 43 per cent reduction respectively in PEPCK activity of the amniotic fluid of the above SIDS-sibling pregnancies, when compared with the normal pregnancies at term. These differences are highly significant. Incidentally, one of those two infants had experienced several periods of apnea in the nursery, lasting approximately 15 seconds each and hence the baby was placed continuously on a respiratory monitor and remained on the monitor at all times, except while feeding. The possibility of eventually developing a prenatal diagnostic test for SIDS, using the above results and the probable genetic implications will be discussed. (Supported in part by NIH Grant # RR08198).

1078435

109 Relationship of Aflatoxins B and G in Aspergillus parasiticus.

J. C. Floyd, J. W. Bennett*, and J. C. Mills, III. Tulane Univ., New Orleans, LA.

In A. parasiticus, the four major aflatoxins (B_1, B_2, G_1, and G_2) are produced simultaneously as products of a secondary biosynthetic pathway that includes the general steps: acetate → anthraquinones → xanthones → aflatoxins. In initial experiments, significant incorporation of unlabeled sterigmatocystin (0.65 μmol) into the major aflatoxins B_1 and G_1 was observed in whole cell experiments with blocked mutants. The versicolorin A-accumulating mutant exhibited 58-66% conversion of sterigmatocystin into aflatoxin B_1, and 4-8% into aflatoxin G_1 after 24-96 hrs incubation in replacement media. The blocked fluff mutant exhibited 12-33% conversion into aflatoxin B_1 and 1-2% incorporation into aflatoxin G_1 under comparable conditions. No such biogenetic conversions were observed when aflatoxins B_1, B_2, G_1 or G_2 were presented to these same mutants. Previous theories implicating aflatoxin B_1 as a precursor of the other aflatoxins must be re-examined in light of these data.

110 Computerized storage, retrieval, manipulation and display of restriction and genetic maps.

Joseph L. Modelevsky*, B.N. Stone**, G. L. Griesinger**, and Franklin H. Norris**.

*Molecular and Cell Biology Research Division, **Scientific Information Systems, Eli Lilly and Co.

Restriction maps are efficient, conventional means for summarizing and communicating descriptions of plasmid constructions and derivatives. Such maps can condense a large amount of information into a single display. Often, entire genetic engineering projects can be described by using a series of restriction map displays.

We describe an integrated set of computer programs designed to permit electronic storage, retrieval, manipulation and display of restriction and genetic maps and supporting textual information. These programs read simple, free format data tables and convert the data tables into sophisticated graphics and alphanumeric displays of restriction maps. The programs test and automatically compensate for overlapping information.

These programs are interactive and communicate in the vernacular of the molecular biologist. Operations include creation of maps, editing of maps, circular color graphics display of maps, alphanumeric display of maps, zooming of subregions, and output to a wide variety of devices.

1078436

111 DETERMINATION OF PLASMID COPY NUMBER BY A SANDWICH HYBRIDIZATION TECHNIQUE

K. Nyberg and I. Palva
Recombinant DNA Laboratory, University of Helsinki, Finland

A novel method for determination of plasmid copy number has been developed. The method is based on a sandwich hybridization technique which utilizes a known gene on the plasmid for hybridization. Two non-overlapping fragments of this gene are cloned into separate vectors. One of the fragments is radiolabeled and used as a probe, while the other is bound to a filter. A three-component, "sandwich" hybrid is formed between the fragments and the complete gene when this is present in the sample. Since the two fragments are non-overlapping, no probe can bind to the filter without the mediation of the correct nucleic acid. Thus the radioactivity attached to the filter is proportional to the concentration of this nucleic acid in the sample.

We utilized this method for determining the copy number of pKTH10, a hybrid plasmid containing the α-amylase gene from *Bacillus amyloliquefaciens* in pUB110. pKTH10 was hybridized between non-overlapping fragments of the α-amylase gene and the copy number determined as a function of cell dry weight and α-amylase production. The host strain was *Bacillus subtilis* BRB1.

Using purified pKTH10 as a standard we were able to show that the amount of radioactivity increased in proportion to the amount of plasmid in the sample. Very little unspecific binding could be detected when no plasmid was present in the sample. No significant difference in hybridization efficiency was observed when solubilized cells of the plasmid-free host strain were added to the standards.

Our results show clearly that the copy number is highest in the early logarithmic growth phase and decreases rapidly with increased cell dry weight and the onset of α-amylase production.

1078437

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Increased Production of K99 Pili under Control of the E. coli Tryptophan Promoter.

Earl R. Shelton, Rick F.H. Salazar, Preston A. Baecker, Steven D. Stoufer, and Hardy W. Chan, Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto CA 94304.

Diarrheal disease of newborn domestic farm animals is caused by the B41 enteropathogenic strain of E. coli which harbors a 78 Kb plasmid capable of expressing the K99 pilus antigen. The pili are required for B41 adhesion to and colonization of the small intestine. We have inserted the E. coli Tryptophan (Trp) promoter into a plasmid containing the genes required for K99 pilus expression and induced the production of increased amounts of K99 pilus antigen suitable for vaccine production.

A 7.0 Kb Bam HI fragment was excised from the 78 Kb plasmid present in the field strain B41 and inserted into pBR322 to produce a recombinant plasmid, pK99-64, which was able to produce normal levels of the K99 pilus antigen in strain HB 101. Insertional gene inactivation with IN5 and the analysis of plasmid encoded proteins using minicells permitted localization of the K99 pilus subunit gene. Identification was confirmed by comparing the amino acid sequence derived from the nucleotide sequence to the known amino-terminal sequence of the protein. To allow overproduction of K99 pili, the E. coli Trp operon promoter/operator was inserted upstream from the K99 pilus subunit gene to produce the recombinant plasmid pBRTrpK99-3.

Addition of excess tryptophan to the culture media completely repressed pilus expression when assayed by either a) slide agglutination of whole cells or b) an ELISA assay with monoclonal antibody directed against the K99 pilus antigen using cell lysates. Up to 10-fold overproduction of pili, compared to pK99-64, was achieved by addition of the tryptophan analogue indoleacrylic acid to the culture media. These pili were partially purified and shown to successfully induce anti-K99 sera upon injection into guinea pigs.

1078438

113 Isolation of a gene that complements an *E. coli hemA* mutation

Anthony Macaluso and Arnold L. Demain
Massachusetts Institute of Technology
Cambridge, MA 02139

The formation of δ-aminolevulinic acid (ALA) by δ-aminolevulinic acid synthetase (ALAS) is the first committed step in the biosynthesis of porphyrins, heme, and vitamin B_{12}. In some bacteria, this step has been shown to be rate limiting in the production of porphyrins. Furthermore, in a vitamin B_{12} producing strain of *Pseudomonas denitrificans*, increased levels of this same enzyme are associated with increased production of vitamin B_{12}. To understand this process at a molecular level, we have cloned an *Escherichia coli* gene that is involved in the synthesis of ALA.

The cloning vector λSE4 (S. Elledge and G. Walker) was used to introduce fragments of *E. coli* chromosomal DNA into a $HemA^-$ *E. coli* strain. $HemA^-$ mutants do not make active ALAS and thus do not grow unless supplemented with ALA. Two colonies that no longer require ALA for growth were isolated following infection with the λSE4 derived clone bank. The recombinant phage from these $HemA^+$ strains contain identical inserts based on restriction analysis.

We have evidence that the gene which complements the *hemA* mutation is under the control of a λ promoter, yet still retains its own regulatory region. Studies are underway to determine if the cloned fragment of DNA encodes the ALAS structural gene or a regulator of ALAS.

1078439

14 Mutations that selectively improve the catalytic activity of glucose isomerase.

B. Ratzkin, C. Fisher and K. Chen. Amgen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.

Enzymes such as glucose isomerase are used in the manufacture of foods because these biocatalysts can carry out specific chemical modifications without the generation of undesirable by-products. Unfortunately, the variety of these enzymes and their particular properties are limited. New enzymes with better properties were usually discovered by finding new microorganisms which is a laborious and time-consuming process. On the other hand, by using recombinant DNA technology, we have undertaken the task of generating a modified enzyme activity. We constructed a plasmid pXK23 which has the XylA gene from E. coli on a 1.6 Kb BglII fragment next to a kanamycin-resistance element. The XylA gene encodes for xylose isomerase an enzyme required for xylose fermentation. We have determined that this enzyme will also isomerize glucose under the appropriate conditions. A fragment carrying the XylA gene and part of the kanamycin-resistance element was isolated from pXK23, mutagenized in vitro and then ligated back into pXK23. A strain which is a XylA mutant and sensitive to kanamycin is transformed to kanamycin-resistance. All of these transformed cells contained the piggy-backed XylA gene. Approximately 1% of the transformants had XylA mutations on their plasmid-born gene. Among these mutants were two which behaved unusually on indicator plates for xylose fermentation. The aberrant behavior of these mutants is dictated by the plasmid. The enzyme encoded by this mutant gene isomerized glucose at a higher rate than the wild type enzyme. The mutations were found to lie within the structural portion of the XylA gene. One mutation was found to be the result of a change in a single base. The result of this study shows that with the appropriate screens one can alter the behavior at random of a particular enzyme. Then one can use these data to predict defined changes in a protein.

1078440

115 TRANSPORT LESIONS ENHANCE PROLINE PRODUCTION BY ESCHERICHIA COLI

R.L. Krogsrud and J.M. Wood, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

Systematic effects to improve fermentation productivity have been designed primarily to amplify biosynthesis and reduce catabolism of desired metabolites. Further increases in chemical yield and simplified recovery procedures should result if microorganisms can be programmed to specifically excrete a valued compound. The movement of molecules across the cell wall may be either passive or mediated. Bacteria possess highly active solute uptake systems or "porters" that maintain intracellular metabolite concentrations 100- to 1000-fold greater than those of the extracellular medium. We are examining proline excretion by proline overproducing strains of Escherichia coli K12 in order to assess the relationship between membrane permeability and proline production. Uncontrolled synthesis of proline is not sufficient to cause its excretion. Bacteria which overproduce proline as a consequence of a genetic defect eliminating regulation of proline biosynthesis possess induced levels of the proline-specific catabolic enzymes and of uptake catalyst, proline porter I. Amino acid limited growth yields bacteria which actively accumulate proline via a second active uptake system, proline porter II. Proline uptake via proline porter I is powered by transmembrane ion gradients; the energy supply for uptake via porter II is under investigation. Genetic lesions that eliminate proline catabolism or either of the proline porters specifically enhance proline excretion by bacteria growing aerobically with D-glucose and ammonium sulfate as carbon and nitrogen sources, respectively. Cytoplasmic proline concentration and membrane permeability are also influenced by medium osmotic strength. The nature of the excretion route and the influence of culture conditions on both transport and productivity are currently under investigation.

116 Properties of a stable, low copy number, amplifiable expression vector

G.O. Humphreys, G. Yarranton, M. Robinson and E. Wright. Division of Molecular Biology, Celltech Limited, 250 Bath Road, SLOUGH SL1 4DY, England.

We have constructed plasmid expression vectors with two origins of replication; that of pSC101 (with the par function) which allows maintenance of the plasmids at 3-4 copies per chromosome, and a ColE1 origin whose natural promoter for RNAII has been replaced by other controllable promoters. When this other promoter is P_R and the plasmid also carries the gene for the thermolabile λ repressor (λcI_{857} gene) the plasmid copy number can be thermally induced. Thus the dual-origin plasmids are totally stable at 30° at 3-4 copies per chromosome, and by shifting the temperature to 37° or above, the copy number can be dramatically increased. Studies of protein expression of genes cloned into these vectors will be presented.

1078441

118 Transposition of IS1 into an Expression Operon for the A Chain of Human Insulin. P. R. Rosteck, Jr.*, F. D. Miller and C. L. Hershberger, Eli Lilly and Company, Indianapolis, Indiana 46285.

Human insulin is prepared by combining the A and B chains purified from separate cultures of *Escherichia coli* K12 containing recombinant plasmids pIA7Δ4Δ1 and pIB7Δ4Δ1, respectively. The insulin peptide chains are synthesized as part of fusion proteins containing portions of the tryptophan operon leader peptide and the E protein joined to the insulin chain by a methionine residue. Tryptophan repression regulates transcription of the artificial operon which initiates at p_{trp}. Translation of the polycistronic mRNA produces a tetracycline resistance protein and the insulin fusion protein. The insulin plasmids exist predominantly as head-to-tail dimers.

A spontaneous derivative of pIA7Δ4Δ1 was identified by electrophoretic analysis of whole cell lysates of cultures producing the A chain. The mutant plasmid differed from the parental dimeric plasmid by the acquisition of a single copy of insertion sequence IS1 within the insulin expression operon. Sequence analysis located the point of integration and revealed an eleven base pair direct repeat of the target site at each end of the inserted element. Transpositional insertion of the strongly polar IS1 sequence inactivated expression of the insulin fusion protein and tetracycline resistance.

1078442

Temperature-Sensitive Regulation of Gene Expression From Plasmids Bearing the Tryptophan Operon Control Region

The tryptophan operon in *Escherichia coli* contains five genes which encode enzymes that participate in the biosynthesis of tryptophan from chorismic acid. Expression of the operon is subject to negative control by a repressor molecule, encoded by the trp R gene, which is not active as a repressor until it has complexed with L-tryptophan (the corepressor). An inactivation or diminution of either component will result in at least partial derepression of the operon.

Two basic methods for inducing the synthesis of heterologous proteins joined to the control region of the tryptophan operon have been reported in the literature: (1) the addition of tryptophan analogs to the culture medium and (2) the dilution of tryptophan in the medium to levels low enough that there will not be sufficient quantities to serve as corepressor. Both of these approaches to derepression have serious disadvantages. The addition of tryptophan analogs can be detrimental to translation and to the normal production of tryptophan by the cell. Dilution of cultures to effect tryptophan starvation is not harmful to the cell, but the magnitude of dilution (10- to 20-fold) required for effective derepression results in a very inefficient use of fermentation vessels.

We have chosen to concentrate on regulating the activity of the trp R gene product, rather than on regulating tryptophan levels, as a method of derepression and have developed a series of expression vectors, bearing the tryptophan operon controlling region, for introduction into the cell line CUV 107 (Reznikoff and Thornton, *J. Bact.* **109**:256) which appears to contain a temperature-sensitive trp R gene product. Cells can be grown to high density at low temperature in tryptophan-rich medium, thus providing ample tryptophan to support normal cell growth and to act as corepressor. Upon raising the temperature of the culture to 42°C, the tryptophan operon is derepressed. This method allows us to grow at least ten times as many cells in a culture vessel as the dilution method, thereby significantly increasing the yield of product.

William J. DeLorbe
David J. Plunkett
Molecular Genetics, Inc.
10320 Bren Road East
Minnetonka, MN 55343

1078443

120 Cloning and Expression of IGFI and IGFII in E. coli

Janet K. Epp and Jo Ann Hoskins
Lilly Research Laboratories, Eli Lilly and Company
307 East McCarty Street, Indianapolis, IN 46285

The insulin-like growth factors (IGF's) or somatomedins are polypeptide hormones which are produced in the liver and circulate in the serum, where their levels are regulated by pituitary growth hormone. The amino acid sequences of IGFI and IGFII are similar and share extensive regions of homology with insulin. The DNA sequences encoding IGFI and II were synthesized chemically (R. Belagaje, IGFI; H. Hsiung, IGFII; unpublished) and were fused to parts of the trpE coding region to create fusion proteins 12 kd, 18 kd, and 29 kd in size. The hybrid genes were cloned into derivatives of pBR322 and pIMIA, a thermoinducible runaway-replication plasmid, behind the trp promoter and ribosome binding site. Thus, we were able to compare the effects of gene size and gene dosage on the level of IGF expression. We observed the highest level of expression, about 30 percent of total cell protein with the 12 kd fusion protein cloned into the runaway-replication plasmid. When expressed at a high level, the 12 kd proteins aggregate and accumulate in a granule and can be isolated to greater than 80 percent purity by differential centrifugation.

121 Role of mRNA Translational Efficiency in Bovine Growth Hormone Expression in Escherichia coli.

BRIGITTE E. SCHONER, HANSEN M. HSIUNG, RAMA M. BELAGAJE, NANCY G. MAYNE and RONALD G. SCHONER, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

The conditions necessary for high-level expression of methionyl bovine growth hormone (met-bGH) in Escherichia coli were investigated. Plasmids were constructed that contain a thermoinducible runaway replicon, and either the E. coli tryptophan or lipoprotein promoter and ribosome binding sites which served as transcriptional and translational initiation sites for the expression of the bGH gene. The expression of met-bGH was very low with either system. However, expression levels of up to 30 percent of total cell protein were obtained following the introduction of additional codons 3' to the intiating AUG codon, thus altering the N-terminal amino acid sequence of bGH. To obtain high-level expression of met-bGH a two-cistron system was constructed in which the codons that enhanced the expression of bGH were incorporated into the first cistron, and the coding region for met-bGH was incorporated into the second cistron. This approach may be generally applicable to achieving high-level expression of a gene that contains N-terminal sequences that do not allow for its efficient expression. Analyses of the stabilities of the bGH derivatives and their transcripts in vivo suggested that the variations in the level of expression were due to variations in the efficiency of mRNA translation.

1078444

122 SELECTION AND CHARACTERIZATION OF INCOMPATIBILITY MUTANTS OF ColE1 RNAI.

*Dooley, Thomas P. and Barry Polisky. Dept. of Biology, Indiana University, Bloomington, Indiana 47405.

The small multicopy bacterial plasmid ColE1 has been extensively studied as a model system for the control of DNA replication and plasmid incompatibility. RNAI has been shown previously to be the major control element of ColE1 replication, and is solely responsible for plasmid incompatibility. Recently we have used a rapid selection to generate mutants of ColE1 RNAI, which have impaired function. Plasmids were constructed previously which have a trp promoter-ColE1 RNAI gene fusion, Ap^r, and an ACYC ori,(replication origin). These constructs such as TF421Δ make functional RNAI, which acts in trans to inhibit replication of the Cm^r plasmid TF345, containing a ColE1 incompatible ori. Therefore cells which contain both plasmids will rapidly lose TF345 when grown in the presence of ampicillin. Mutagenesis of TF421Δ by nitrosoguanidine or mutD5, followed by transformation into cells containing TF345, and selection for Ap^rCm^r colonies has allowed us to select mutants of TF421Δ. These incompatibility mutants have been analyzed by DNA and RNA sequencing, in vitro transcriptions, segregation analysis, and other methods. These point mutants are of various classes; promoter down mutants, transcription termination mutants, RNAI loop mutants, and various novel mutants in the 5' end of the RNAI molecule. This system allows us to determine the critical structural and functional domains of ColE1 RNAI.

1078445

Examination of Calf Prochymosin Accumulation in *Escherichia coli*: Disulfide Linkages are a Structural Component of Prochymosin-Containing Inclusion Bodies.

J.M. Schoemaker[1] and F.A.O. Marston.
Celltech Ltd., Slough, U.K.

Abstract

Recent reports have shown that synthesis of certain recombinant proteins in *Escherichia coli* results in the production of intracellular inclusion bodies. These studies have not analyzed the structure of the inclusion especially regarding the intermolecular forces holding it together. This report has examined structural aspects of inclusion bodies made in *Escherichia coli* as a result of high level expression of the eukaryotic protein, calf prochymosin. Prochymosin is a monomeric protein containing three disulfide bridges. It was expressed at up to twenty percent of cell protein from a plasmid containing the *Escherichia coli* trptophan promoter, operator, and ribosome binding site. Proteins in the inclusion bodies were analyzed by Western blotting of sodium dodecyl sulfate/polyacrylamide gels. When experiments were done using conditions which preserved the *in vivo* state of thiol groups, inclusions were shown to be composed of multimers of prochymosin molecules which were interlinked to a large extent by disulfide bonds. The inclusion bodies also contained a high concentration of reduced prochymosin monomer which may be primarily bound to the surface of the body, in contact with the reducing environment of the cytoplasm. The presence of intermolecular disulfides probably contributes to the difficulty of solubilizing recombinant prochymosin during its purification from *Escherichia coli*.

Present address: [1]Department of Biochemistry and Molecular Biology
The University of Chicago
Chicago, Illinois 60637

1078446

Cloning and expression of alpha-galactosidase for use in beet sugar industry

Dr. Ralf Mattes, Boehringer Mannheim GmbH, Forschungszentrum Tutzing, Bahnhofstr. 9-15, 8132 Tutzing, FR Germany

The enzyme alpha-galactosidase is an important tool for removing raffinose contaminations in beet sugar molasses and thereby improving yield and purity of crystallized sucrose. The enzyme splits raffinose into galactose and sucrose.

Previous enzyme preparations from various fungi revealed one or more of the following process disadvantages:

Presence of invertase, unfavourable pH optimum, entrapment of enzyme in mycelial pellets, low specific activity, need for inducer during fermentation, etc.

Using alpha-galactosidase from raffinose-utilizing bacteria, a more favourable pH is possible. The genetic analysis of raffinose genes of E.coli revealed that these bacteria also produce invertase and a specific transport protein. The organisation of these raf-genes within a raf-operon under negative regulation was verified by transposon mutagenesis and cloning of the raf-operon, which allowed the construction of a pHysical map. Subcloning lead to the selective expression of alpha-galactosidase essentially free of invertase.

The application of enzyme preparations in beet sugar molasses was successfull in large scale.

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CLONING OF A GENE FOR ARYLETHERASE - AN ENZYME INVOLVED IN LIGNIN DEGRADATION

K. NARVA, J. CARY, Y. CHONG, AND V.R. SRINIVASAN
Department of Microbiology, Louisiana State University
Baton Rouge, Louisiana 70803

Lignin comprises about one-fourth of the wood tissue of plants and is one of the most abundant readily available renewable organic resources. It is a three dimensional branched polymer biosynthesized essentially from nine-carbon units possessing phenylpropane skeletons. The macromolecule is formed by covalent linking of the monomers mainly through three different bonds namely aryl-alkyl ether, aryl-aryl ether and diphenyl, of which aryl-alkyl ether bonds are the most abundant. Studies were designed to isolate an organism having the potential to cleave aryl-alkyl ether bonds. A soil organism was enriched on lignin from Kraft Black Liquor as the sole carbon source and tentatively identified as *Erwinia* spp. The gene encoding an aryletherase from this organism was cloned into the Bam HI site of *E. coli* plasmid pBR322. *E. coli* transformants (amp^R tet^S) with this plasmid were selected on M9 salts and ampicillin minimal media with salicin as the sole carbon source (on the assumption that hemiacetal linkage may be analogous to a psuedo-ether linkage). The clone has been shown to degrade a number of compounds containing arylether bonds inclusive of Kraft lignin. The recombinant plasmid (pNC1) contains an insert of approximately 810 bp of *Erwinia* as shown by nucleic acid hybridization. Partial restriction endonuclease mapping of the insert has been carried out. The gene product is apparently secreted from *E. coli* transformed with pNC1 since most detectable enzyme activity is found in the culture supernatant.

1076448

PROGRAM ABSTRACT

126 Aldose Reductase(s) in the Yeast *Pachysolen tannophilus*: Purification and Characterization [1]

P. L. Bolen, J. A. Bietz, and R. W. Detroy

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Although many yeasts assimilate xylose, few are able to efficiently ferment xylose to ethanol. One of the earliest identified with this capacity was *Pachysolen tannophilus*. Smiley and Bolen (1983) have shown cell extracts of D-xylose-grown *P. tannophilus* to convert D-xylose to xylitol in a reaction catalyzed by an NADPH-requiring D-xylose reductase (aldose reductase E.C. 1.1.1.21). The same cell extracts were shown to oxidize xylitol to D-xylulose in an NAD-linked reaction catalyzed by the enzyme xylitol dehydrogenase (D-xylulose reductase E.C. 1.1.1.9).

Recently, we reported that growth of *P. tannophilus* on either L-arabinose or D-galactose, like growth on D-xylose, leads to the induction of both NADPH-linked D-xylose reductase and NAD-linked xylitol dehydrogenase activities (Bolen *et al.*, 1984, Biochem. Bioeng., in press). Furthermore, L-arabinose and D-galactose were found to serve as substrates for NADPH-linked reactions in extracts of cells grown on either D-xylose, L-arabinose, or D-galactose. The products of these NADPH-linked L-arabinose and NADPH-linked D-galactose reactions were shown to be L-arabitol and galactitol, respectively. However, unlike xylitol, neither L-arabitol nor galactitol would serve as substrates for NAD-linked reactions. We concluded that divergence of the metabolic pathways of D-xylose, L-arabinose, and D-galactose must occur following formation of the alcohol, but the question of whether the NADPH-linked reactions were carried out by one or more enzymes remained unresolved.

[1] To be presented at the American Society for Microbiology Conference on Genetics and Molecular Biology of Industrial Microorganisms, Bloomington, Indiana, September 30-October 3, 1984.

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To examine this question, we have undertaken purification of the NADPH-linked D-xylose reductase from P. tannophilus. Fractionation of D-xylose-grown P. tannophilus cell extracts by affinity chromatography and high performance liquid chromatography (HPLC) over an anion exchange column results in the isolation of a single protein band, judged by SDS polyacrylamide gel electrophoresis and reverse phase HPLC, that retains ability to reduce D-xylose, L-arabinose or D-galactose when supplied either NADPH or NADH. An antibody preparation against this 37,000 kd fraction yielded a single band in western blots that correlated with induction of activity in extracts of cells grown under a variety of conditions. Partial amino acid sequence data (64 residues) from the N-terminus failed to reveal evidence of heterogeneity of sequence. Thus, we conclude this protein band to either be a single enzyme, an aldose reductase (E.C. 1.1.1.21), or very similar isozymic forms of an enzyme of this type. With either NADPH or NADH as cofactor, specific activity was greatest with L-arabinose and poorest with D-galactose.

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Production of Active Xylose Reductase in *Pachysolen tannophilus* is Temperature Sensitive[1]

N. J. Alexander

Northern Regional Research Center, Agricultural Research Service,

U.S. Department of Agriculture, Peoria, Illinois 61604

NADPH-linked xylose reductase and NAD-linked xylitol dehydrogenase are inducible enzymes in *Pachysolen tannophilus* when the cells are grown on xylose at 30° C. However, when grown on xylose at 37° C., there is little detectable xylose reductase activity present in the cell extract. No protein of the molecular weight of xylose reductase can be found in the cell extract, as detected by coomassie blue staining of polyacrylamide gels. However, in an *in vitro* assay system, small amounts of an intermediary product, xylitol, are formed by 37° C. xylose-grown cells, suggesting that small quantities of NADPH-dependent reductase are present and functional. It is apparent that induction of a significant quantity of an active form of this enzyme is temperature sensitive. The growth rate of *P. tannophilus* on xylose at 37° C. is about one-half that at 30° C. and ethanol production is about the same at both temperatures. These results suggest that either another pathway exists in *P. tannophilus* for utilization of xylose or that small quantities of xylose reductase are sufficient for growth and fermentation processes.

[1] To be presented at the American Society for Microbiology Conference on Genetics and Molecular Biology of Industrial Microorganisms, Bloomington, Indiana, September 30-October 3, 1984.

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COMPARISON OF "KILLER" PHENOTYPE IN *Saccharomyces cerevisiae* AND *Kluyveromyces lactis* YEASTS

C.J. Panchal, C. Meacher, G.G. Stewart
Production Research Department
Labatt Brewing Company Limited
London, Ontario, Canada

The secretion of killer toxins by some strains of yeasts is a phenomenon of significant industrial importance. In addition to presenting a model of protein secretion (the killer toxins being true extracellular proteins), the killer yeasts are of great use in fermentations susceptible to contamination by unwanted yeast strains. The activities of two killer yeast species, *Saccharomyces cerevisiae* (RNA plasmid coded toxin) and *Kluyveromyces lactis* (DNA plasmid coded toxin) against sensitive cells on agar plates containing different sugars at two different pH values were investigated. The toxic effect of the *Saccharomyces* killer strain was quite evident when assays were performed on methylene blue agar plates containing either glucose, fructose, galactose, maltose or glycerol at pH 4.5. While there was some killer activity at pH 6.5 with the sugars glucose and fructose, no activity could be detected with either galactose, maltose or glycerol. The *Kluyveromyces* killer activity, on the other hand, was very low at pH 4.5 with the sugars glucose, fructose or galactose. Enhanced activity could be found at pH 6.5 with galactose, maltose or glycerol. Glucose and fructose were found to be poor substrates for detecting killer activity at pH 6.5. Surprisingly however, with maltose as the carbon source, *Kluyveromyces* killer activity could be detected at both pH 4.5 and 6.5.

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Labatt Brewing Company Limited
London, Ontario, Canada

The secretion of killer toxins by some strains of yeasts is a phenomenon of significant industrial importance. In addition to presenting a model of protein secretion (the killer toxins being true extracellular proteins), the killer yeasts are of great use in fermentations susceptible to contamination by unwanted yeast strains. The activities of two killer yeast species, *Saccharomyces cerevisiae* (RNA plasmid coded toxin) and *Kluyveromyces lactis* (DNA plasmid coded toxin) against sensitive cells on agar plates containing different sugars at two different pH values were investigated. The toxic effect of the *Saccharomyces* killer strain was quite evident when assays were performed on methylene blue agar plates containing either glucose, fructose, galactose, maltose or glycerol at pH 4.5. While there was some killer activity at pH 6.5 with the sugars glucose and fructose, no activity could be detected with either galactose, maltose or glycerol. The *Kluyveromyces* killer activity, on the other hand, was very low at pH 4.5 with the sugars glucose, fructose or galactose. Enhanced activity could be found at pH 6.5 with galactose, maltose or glycerol. Glucose and fructose were found to be poor substrates for detecting killer activity at pH 6.5. Surprisingly however, with maltose as the carbon source, *Kluyveromyces* killer activity could be detected at both pH 4.5 and 6.5.

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The supernatant (containing killer toxin) from *Kluyveromyces lactis* broth was found to have activity at temperatures up to 55°C, after which activity was rapidly lost. It was also found to be more lethal to the α-factor super sensitive yeast strains (sst$^-$) as compared to a normal α-factor sensitive strain (SST). The lethality of the *Kluyveromyces* toxin against *a* type *Saccharomyces* strains as well as some α type cells (including 'killer' cells) suggests that the *Kluyveromyces* toxin has some effects similar to *Saccharomyces* α-factor and other effects more lethal than those of α-factor.

129 Expression in yeast of tissue-type plasminogen activator under the control of the promoter for repressible acid phosphatase (PHO5)

J. Heim, F. Barth, M. Gruetter, H.J. Treichler, B. Meyhack, A. Hinnen; Department of Biotechnology Ciba-Geigy AG, CH-4002 Basel, Switzerland

The gene for tissue-type plasminogen activator (TPA) from Hela cells has been expressed in yeast (S. cerevisiae) under the control of the PHO5 promoter. The DNA construction includes the PHO5 promoter, the complete signal sequence of the repressible acid phosphatase joined in frame to the mature coding sequence for human TPA and a DNA fragment containing the PHO5 transcription termination signals (A). Alternatively, the signal sequence was removed and the mature TPA coding sequence was directly joined to the PHO5 promoter region using a synthetic oligonucleotide linker (B). Synthesis of TPA is in both cases induced by low concentrations of P_i in the medium.

Construction (B) codes for a protein which is immunologically present, functionally however almost inactive. (A) results in enzymatically active TPA which can be purified from cell extracts by affinity chromatography after dissociation of the internal membranes. The eluted TPA is mostly in the one chain form with an apparent molecular weight of 67 000. Purified recombinant TPA from yeast has a similar specific activity, it binds to fibrin and is stimulated by fibrin as genuine TPA from melanoma cells.

130 Stimuli that induce a yeast heat shock gene fused to beta-galactosidase.

C. BRAZZELL and T. INGOLIA, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

Yeast contains a multigene family related to the Drosophila heat shock gene hsp70. Two members of this family, YG100 and YG101, have been previously characterized and only YG100 was found to have elevated levels of transcription after heat shock. The yeast hsp70 genes contained on YG100 and YG101 were truncated and fused to the E. coli lacZ gene contained on pMC1587. The resulting plasmids directed synthesis of the beta-galactosidase gene as measured by in vitro enzyme assays and by colorimetric assays on plates. The expression level from the YG101 gene was constant under all the conditions tested, whereas expression driven by the YG100 gene could be induced over fifty fold. Other stimuli besides heat, including recovery from anoxia and high cell density, were found to strongly induce YG100 gene expression. Most physical and chemical stimuli tested, including UV irradiation, zymolyase treatment, and ethanol, did not stimulate expression of this heat shock gene.

131 Expression of Heterologous Genes in *Saccharomyces cerevisiae* from Vectors Utilizing the Glyceraldehyde-3-Phosphate Dehydrogenase Gene Promoter.

Kevin M. Egan and Grant A. Bitter, Amgen, Thousand Oaks, CA 91320.

The promoter region from the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) gene has been characterized. A 652 bp TaqI restriction fragment which includes the transcription start site and 15 bp of untranslated leader coding region was cloned into M13mp9 from which it may be excised as a HindIII-BamHI portable promoter. The entire DNA sequence of the GPD promoter has been determined. Generalized expression vectors employing the GPD portable promoter have been constructed which incorporate the entire yeast 2μ plasmid cloned at the EcoRI site in the large unique region. In such constructions, the REP1 and REP2 genes, as well as the REP3 locus, remain intact and the plasmids thus amplify to high copy numbers and are stably maintained in cir° yeast hosts. A number of heterologous proteins have been produced utilizing these expression vectors including hepatitis B surface antigen, consensus α-interferon and γ-interferon. The 5' end of the HBsAg gene was replaced with a chemically synthesized segment which restored the native GPD untranslated leader and utilized optimal yeast codons for the first 32 amino acids. This modification yielded a 10-15 fold increased expression resulting in HBsAg synthesis as 1-2% of total cell protein. A completely synthetic IFN-γ gene utilizing optimal yeast codons (codon bias index = 0.99) is expressed from these vectors as 5-10% of the total cell protein.

1078455

132 Multiple cytochrome P-450 proteins from Saccharomyces cerevisiae and Candida Tropicalis: immunological comparisons. C. CHEN, C.R. DEY AND J.C. LOPER, Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH.

Mammalian cytochrome P-450 monooxygenase systems have been characterized extensively for their essential roles in sterol synthesis and in the biotransformation of xenobiotic compounds. Similar functions have been documented for P-450 systems in yeasts, particularly demethylation of lanosterol as a step in ergosterol biosynthesis, studied in Saccharomyces cerevisiae; and n-alkane hydroxylation, a substrate-inducible reaction in yeasts that grow on such hydrocarbons for carbon and energy. As part of a genetic analysis of yeast cytochromes P-450, we have isolated protein components and developed polyclonal antibodies in rabbits. S. cerevisiae strains and Candida tropicalis ATCC 750 were grown by fermentation in high levels of glucose and the latter organism was also cultured on tetradecane. NADPH-cytochrome P-450 reductase preparations purified from strain 750 grown under both conditions showed the same apparent m.w. and were inhibited by antibody produced against one of them, thus these reductases are from a single gene or closely related genes. Reductase from S. cerevisiae was not inhibited by that antibody and did not cross react with it in Western immunoblots. Antibody produced against the major cytochrome P-450 of one S. cerevisiae strain identified different molecular weight variants of P-450 from two other S. cerevisiae strains and cross reacted weakly with P-450 from glucose grown 750 cells. None of these P-450 proteins reacted with antibody to cytochrome P-450 purified from tetradecane grown 750 cells. These antibodies will be useful for isolation and characterization of genes for yeast cytochrome P-450 systems. Supported by a grant from USEPA.

1078456

133 CONSTRUCTION OF PLASMIDS CONTAINING C. ACREMONIUM RIBOSOMAL DNA,

P. L. Skatrud, L. Carr, and S. W. Queener, Eli Lilly and Company, Indianapolis, IN 46285

A recently developed transformation system for C. acremonium produces transformants at a low frequency by integration (See Abstract #30, this meeting, "Recombinant DNA Studies in Cephalosporium", S. W. Queener et. al.). A higher frequency of transformation would be advantageous in further optimization of transformation conditions and isolation of important genetic elements from the genome of C. acremonium. Szostak and Wu (Plasmid 2:536-554, 1979) demonstrated a marked increase in transformation frequency of yeast when fragments of yeast ribosomal DNA (rDNA) were included in the transforming plasmid.

The plasmid pYlrA12, which contains the entire rDNA repeat of Saccharomyces cerevisiae, was used as a probe to locate the rDNA of C. acremonium. Initially, to demonstrate the appropriateness of the probe, total C. acremonium DNA was digested to completion with several restriction enzymes, blotted to nitrocellulose, and hybridized with pYlrA12. The entire rDNA repeat of C. acremonium was located on a 9.2 kb Pst1 restriction fragment. A C. acremonium gene library (prepared in the lambda phage Charon 28 by P.L. Skatrud, S. E. Fishman, P. R. Rosteck, Jr., and C. L. Hershberger) was screened for sequences which hybridized with the yeast rDNA. Four phage containing rDNA were purified. Restriction endonuclease cleavage patterns and Southern hybridization analysis confirmed the source of the newly acquired rDNA to be C. acremonium.

Several restriction fragments containing C. acremonium rDNA were subcloned into the plasmids pIT221 and pPS5. pIT221 contains a fragment of C. acremonium mitochondrial DNA which functions as an autonomous replication sequence (ARS) in yeast. pPS5 has the mitochondrial DNA sequence deleted. These plasmids, with various degrees of homology to the C. acremonium chromosomal genome and/or mitochondrial genome can now be compared for their ability to transform C. acremonium to hygromycin resistance (See Abstract #134 "Phosphotransferase Activity in Cephalosporium acremonium", J. L. Chapman and S. W. Queener.

1078457

134 PHOSPHOTRANSFERASE ACTIVITY IN CEPHALOSPORIUM ACREMONIUM. J. Chapman and S. W. Queener, Eli Lilly and Company, Indianapolis, IN 46285

Integrative transformation of C. acremonium has been achieved using hygromycin B resistance as the phenotypic marker. The transformation protocol and detailed analysis of one transformant, CPC-T1, are described in "Recombinant DNA Studies in Cephalosporium" by S. Queener et. al. presented at this meeting. (See Abstract #30). Modifications of the vector (pIT221) used in these transformations are also reported (See Poster and Abstract #133, "Construction of Plasmids Containing C. acremonium Ribosomal DNA" by P. Skatrud et. al.)

As an independent confirmation of transformation of C. acremonium, enzyme assays were conducted to demonstrate the presence of the hygromycin phosphotransferase gene product in the transformant. Hygromycin phosphotransferase activity was detected in the CPC-T1 transformant, whereas no activity was detected in the untransformed parent strain ATCC 11550. An extract from a Streptomyces ambofaciens strain known to contain the gene on a multi-copy plasmid was used as a positive control; the activity from this extract was an order of magnitude greater than that demonstrated in the CPC-T1 extract. This difference is apparently due to the low copy number of the gene integrated in the chromosome of CPC-T1 compared to the high copy number in the S. ambofaciens cells.

This S. ambofaciens extract was also used as a control in the substrate specificity testing. In this control as well as in the CPC-T1 extract, it was demonstrated that the enzyme phosphorylated only hygromycin B and not other related aminoglycosides.

1078458

201 Characterization of tyrosinase synthesized by *Streptomyces antibioticus* transformed with pIJ702. Wayne Herber and Edward Katz, Georgetown University, Washington D.C.

The structural gene coding for the enzyme, tyrosinase (*Streptomyces antibioticus*), has been cloned and the nucleotide sequence determined using the dideoxy method of Sanger *et al*. (Bernan *et al*., this meeting). The synthesis of intracellular and extracellular enzyme has been investigated in twenty strains of *S. antibioticus* transformed with the high copy number plasmid, pIJ702, which confers thiostrepton resistance (*tsr*) and the *mel*$^+$ phenotype. Transformed strains grow less vigorously but exhibit a 2 to 3-fold increase in levels of secreted enzyme as well as a 50 to 150-fold enhanced intracellular activity in comparison with parental strains of *S. antibioticus* (lacking pIJ702). Exported enzyme in the parent comprises greater than 95% of total tyrosinase activity in contrast to 20% or less in strains harboring pIJ702. *S. antibioticus*, transformed with the high copy plasmid pIJ350 (carries only the *tsr* determinant), produced levels of both extracellular and intracellular tyrosinase activities similar to parental strains.

Intracellular tyrosinase has been purified (185-fold) to homogeneity from extracts of *S. antibioticus* containing pIJ702 by means of protamine sulfate precipitation, ammonium sulfate fractionation and column chromatography (DEAE-cellulose, Sephadex G-75 and Phenyl-Sepharose). The molecular weight of the intracellular enzyme was found to be 29,500 daltons based on analysis by SDS-PAGE and gel filtration chromatography. This data confirms the predicted value (29,430 daltons) derived from the nucleotide sequence of the structural gene for tyrosinase. Kinetic parameters of the intracellular enzyme have been examined.

1078459

202 Nucleotide Sequence of the Tyrosinase Fragment from *Streptomyces antibioticus*.

Valerie Bernan, David Filpula and Edward Katz, Georgetown University, Washington, D.C. and Genex Corporation, Gaithersburg, Md.

The *Bcl*I-cleaved DNA fragment (1.56 kb) containing the structural gene coding for the enzyme, tyrosinase (*Streptomyces antibioticus*; Katz *et al*., J.G.M. 129, 2703, 1983), was sequenced by the dideoxy method of Sanger *et al*. Molecular weight (30,325 daltons) and amino acid sequence of the enzyme were determined from analysis of the nucleotide sequence of the cloned structural gene (IV; 819 b.p.). The sequence homology between the amino terminus of the *S. glaucescens* tyrosinase (Crameri *et al*., J.G.M. 128, 371, 1982) in comparison with the predicted N-terminal sequence of the enzyme from *S. antibioticus* is striking:

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                                Glu     Thr             Asp
Met-Thr-Val-Arg-Lys-Asn-Gln-Ala-Ser-Leu-Thr-Ala-Glu-Glu-Lys-Arg-Arg-Phe-Val-Ala-
ATG ACC GTC CGC AAG AAC CAG GCG TCC CTG ACC GCC GAG GAG AAG CGC CGC TTC GTC GCC
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Examination of the nucleotide distribution in codon triplets of the structural gene revealed a high G/C content in the first and third positions, characteristic of other *Streptomyces* genes studied. A second open reading frame (II; 348 b.p.) upstream from the structural gene suggests a possible regulatory region since insertion of foreign DNA at the *Sph*I or *Bgl*II restriction sites inactivates expression of the structural gene. 5' to this region is a sequence (I; 228 b.p.) that may contain the -10 and -35 promoter sites required for initiation of RNA transcription. A 162 b.p. sequence (III) flanked by IV (3') and II (5'), contains recognition sites for *Sst*I, *Sst*II, and *Sal*GI. Cloning into the unique *Sst*I site also inactivates the structural gene. It is concluded that the nucleotide sequence of the tyrosinase fragment contains natural initiation signals for transcription and translation.

1078460

203 Expression of the *Streptomyces azureus* thiostrepton resistance gene in *Bacillus subtilis*

Guy G. Hoffman and Ulfert Hornemann, School of Pharmacy, University of Wisconsin, Madison, WI 53706

The *S. azureus* thiostrepton resistance gene was inserted into the erythromycin resistance gene present together with a chloramphenicol resistance gene in plasmid pHWI (S. Horinouchi, B. Weisblum, J. Bacteriol, 150, 804-825, [1982]) which is derived from *Staphylococcus aureus* plasmids. The construct was transformed into *B. subtilis*. The *B. subtilis* transformants obtained initially expressed only chloramphenicol resistance despite the fact that plasmids reisolated from them (designated pBV 101 and pBV 102) carried the desired insert in both expected orientations. Plating massive amounts of both kinds of transformants on 0.5 μg/ml thiostrepton and 10 μg/ml chloramphenicol yielded a few colonies in each case. Among pBV 102 carrying cells but not among those containing pBV 101 were found two colonies able to grow on 50 μg/ml thiostrepton and 10 μg/ml chloramphenicol. Restriction endonuclease analysis of the plasmids (pBV 103 and pBV 104) carried by these cells revealed that DNA deletions had occurred which bring the open reading frame sequences of the thiostrepton resistance gene (the sequence was kindly made available by Dr. M. Bibb) in close proximity to the promoter of the erythromycin resistance gene. It is thus possible that the thiostrepton resistance gene is read from this promoter, however, direct proof for this assumption is not yet available. Both plasmids were fused to plasmid pBV 404 which carries the neomycin phosphotransferase gene and which is derived from plasmid pIJ 702. The resulting chimeric plasmids were transformed into *Streptomyces lividans* and thiostrepton and neomycin resistant colonies were recovered. Plasmids isolated from these showed large deletions of *S. aureus* DNA sequences precluding successful retransformation of *B. subtilis*.

1078461

204 STREPTOMYCES GRISEUS PLASMID pSG1

Yair Parag[1], Dror Bar-Nir[2], Maria E. Goedeke[1] and Amikam Cohen[2]
Departments of Genetics[1] and Molecular Genetics[2]
The Hebrew University, Jerusalem, Israel

A plasmid of a length of 16.6 kb -pSG1 - has been isolated from chloramphenicol resistant derivatives of Streptomyces griseus NRRL 3851, a cephamycin producer, following transformation of protoplasts with unrelated plasmids. Southern hibridization experiments with radioactive probes prepared of pSG1 fragments and immobilized S. griseus DNA fragments indicated that the plasmid is present in the progenitor strain in a chromosomally integrated state. In the pSG^+ isolates plasmid sequences exist both as integrated sequences and as free plasmids. The integrated state of maintenance persists in strains which have been cured of the free plasmid. The junction site on the plasmid is located on a 0.5 kb EcoRI-SalI fragment. The chromosomal integration site was demonstrated to be the same in all strains which were derived of S. griseus NRRL 3851. The occurence of both states of plasmid maintenance in the same clones indicates an integrated pSG1 sequence does not interfere with the free plasmid replication and partition and suggests that the establishment of the free state may involve a replicative exision of pSG1 from S. griseus chromosome. Segregation of $pSG1^+$ vs $pSG1^-$ in conjugation experiments indicates that this lack of interference is chromosomally controlled. The replication excision is probably induced by the transformation procedure. It is thus suggested that this can be a general method for obtaining plasmids in apparently plasmid-less strains of streptomycetes. The coexistance of free plasmids and chromosomally integrated plasmid-sequences can be used for cloning of foreign genes into the chromosome via the plasmid following homologous recombination. The integration site was narrowed down to within the segment of HinCII-SalI of 0.4 kb. This segment has been cleaved to smaller fragments and is now being sequenced. The plasmid pSG1 appears also with reiterated sequences, over 15 repeats; up to now four different-size segments - 3.8 to 5.4 kb - were found to be involved in the reiteration in different clones, all four are derived from the same region of the plasmid.

1078462

205 Loss of activity in a streptomycete and simultaneous appearance of a plasmid. R. MOROSOLI, D. Kluepfel, F. Shareck. Centre de recherche en bacteriologie, Institut Armand-Frappier, Université du Québec, Laval, Qué., H7N 4Z3

Curing agents such as ethidium bromide or heat treatement induced total loss of cellulolytic activity in a Streptomyces strain; similarly, although at lower frequency, some spontaneous loss was also observed. No reversion of the cellulase-negative mutants was found. Isolation of chromosomal and plasmid DNA was carried out on wild-type and mutant strains and compared by agarose gel electrophoresis. The same self replicating 8.8kb plasmid was detected in all examined mutants, but no plasmid like materiel was found in the wild strain. Hybridization experiments of plasmid DNA indicated that the plasmid sequence originated from the chromosomal DNA and that this particular sequence was present at a single copy level in the wild strain. The possible role of this plasmid will be discussed.

206 EVIDENCE FOR A SEX FACTOR IN STREPTOMYCES ERYTHREUS
Janet DeWitt, Abbott Laboratories, North Chicago, IL 60064

Streptomyces erythreus was found to contain a transmissable plasmid SEP1 which acts as a sex factor. Recombination occurred between strains containing single and multiple markers at a frequency of 10^{-7} to 10^{-5} during mating on agar. A strain, JD101, isolated from a spo$^-$ sector of a normal colony was found to be lethal zygosis sensitive. Pocks were formed when spores of the parental type were plated on a lawn of JD101 indicating the loss of a transmissible plasmid, SEP1, from this strain. Recombination did not occur between derivatives of JD101 lacking SEP1, but did occur if SEP1 was transferred to one of these strains, or if these strains were crossed with other strains containing SEP1. SEP1 could also be transferred at high frequency between strains. This data is consistant with SEP1 acting as a fertility factor in S. erythreus. SEP1 could not be detected in agarose gels or $CsCl_2$ gradients using standard plasmid isolation procedures. Interspecific crosses are now being made to transfer SEP1 into S. lividans to study its properties in standard S. lividans crosses.

1078463

207 Characterization of shuttle plasmid vectors suitable for the identification of promoters or terminators in Streptomyces. S. KUHSTOSS, R. BELAGAJE, HANSEN HSIUNG and R. N. RAO, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

We have constructed a plasmid vector (pKC356) that can replicate in Escherichia coli K-12 and in Streptomyces ambofaciens. The vector can be selected in E. coli by ampicillin resistance and in S. ambofaciens by thiostrepton resistance. The vector carries Tn5 neomycin resistance gene without its promoter, and this feature has been used to show that p_{neo} and λp_L are functional in S. ambofaciens. Starting from plasmid pKC388 in which Tn5 neomycin resistance gene is expressed from its cognate promoter, we have functionally characterized two DNA segments, one a synthetic sequence and another from Streptomyces phage, ΦC31, that interfere with the expression of neomycin resistance gene in both E. coli and S. ambofaciens. Deletion of a stem structure in the synthetic sequence restored the expression of neomycin resistance. The ΦC31 sequence was initially isolated in an E. coli terminator probe plasmid. From these experiments we suggest that these sequences are acting as transcriptional terminators.

208 Cloning of Streptomyces DNA in shuttle cosmid vectors and their characterization. R. STANZAK and R. N. RAO, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

We have constructed three cosmid vectors (~11kb in size) that can shuttle between Escherichia coli and Streptomyces. These vectors carry three λ cos sites with a unique restriction endonuclease site between the first and the second cos site. A unique BamHI site present in the plasmid is the site used for cloning. Our earlier experiments have shown that E. coli apramycin resistance gene and Tn5 neomycin resistance gene are expressed in Streptomyces. All these vectors can be selected in E. coli by ampicillin resistance or apramycin resistance or neomycin resistance and in Streptomyces by apramycin or neomycin resistance. These vectors have been used to generate a cosmid library (~5,000 independent clones) from Streptomyces felleus. Four random cosmids containing inserts were characterized by restriction enzyme analysis and DNA blots. The insert size varied from 34 - 39 kb. The cloned DNA hybridized to S. felleus chromosomal DNA and was colinear with it. The cosmid library is being used to isolate specific cosmid clones by genetic complementation and by specific DNA probing.

209 Streptomyces plasmid SCP2* and DNA cloning vectors derived from it.

D. J. LYDIATE, F. MALPARTIDA, T. KIESER and D. A. HOPWOOD
John Innes Institute, Norwich, U.K.

SCP2* is a 30 kb low copy plasmid isolated from Streptomyces coelicolor A3(2). Like most Streptomyces plasmids it is self-transmissible, produces pocks and confers fertility to its host. It can stably coexist (is compatible) with the plasmids pIJ101 and SLP1.2 and their derivatives. SCP2*-derived DNA cloning vectors and lysogenic phages are currently the only low copy number cloning vectors available for Streptomyces. SCP2*-derived vectors are known to support the stable replication of large (greater than 30 kb) DNA inserts which are needed for the cloning of whole metabolic pathways such as those for antibiotic biosynthesis (see F. Malpartida and D. A. Hopwood, Nature 309-462 (1984)). They may also be useful for the cloning and study of regulatory genes and for genes which are deleterious to their host when present in high copy number. The host-range of derivatives of SCP2*, originally thought to be limited to S. coelicolor A3(2) and its close relatives, is much wider; many strains have been successfully transformed with and found to maintain these plasmids.

As a basis for the construction of plasmid vectors, a detailed restriction map of SCP2* has been constructed and plasmid functions including replication, transfer, pock formation and stability have been localised on it. About 12.5 kb (40%) of SCP2* can be deleted or disrupted by insertions without effect on any known plasmid functions and sites within such segments are therefore presumed to be available for cloning. Single cloning sites for BamHI, BglII, EcoRI, HindIII, SstI, XbaI and XhoI are available on various SCP2* derivatives.

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210 A 2.6 kb DNA sequence of Streptomyces coelicolor A3(2) which may function as a transposable element

D. J. LYDIATE, H. IKEDA* and D. A. HOPWOOD

John Innes Institute, Norwich, U.K.

*Present address: School of Pharmaceutical Studies, Kitasato University, Tokyo, Japan

S. coelicolor A3(2) contains 2.6 kb CCC DNA molecules at a copy number of less than 0.2 per chromosome, which is unusually low for an autonomous plasmid. This DNA does not have homology with any of the previously identified plasmids of S. coelicolor, namely, SCP1, SCP2, SLP1 and SLP4. Two linear copies of the 2.6 kb sequence per host genome are also present in S. coelicolor integrated into a larger replicon (presumably the chromosome). Both copies have a similar or identical linear arrangement, and have been preserved through several branches of the pedigree of S. coelicolor A3(2) derivatives. It is therefore unlikely that free circular molecules arise by simple excision of the integrated copies. S. lividans 66 and S. violaceolatus ISP5438 (both of which are closely related to S. coelicolor A3(2), together with 12 more distantly related Streptomyces strains, showed no sequences homologous with the 2.6 kb sequence, suggesting its "recent" acquisition by S. coelicolor A3(2). Despite the absence of homology between S. lividans 66 DNA and the 2.6 kb sequence, a ϕC31 phage derivative lacking its attP site (and therefore unable to integrate into the host genome by its normal mechanism) was able to lysogenise S. lividans 66 when the 2.6 kb sequence was cloned into it.

10784466

211 Shuttle Vectors Containing the SCP2* Replicon. J.L. Larson* and C.L. Hershberger, Eli Lilly and Company, Indianapolis, Indiana 46285.

We described a 5.9 kb EcoRI-SalI restriction fragment that contains the minimal replicon of the 31 kb plasmid SCP2* (J. Larson and C.L. Hershberger, J. Bacteriol. 157:314-317, 1984). Shuttle vectors pHJL197 and pHJL198 for streptomycetes and Escherichia coli contain the 5.9 kb streptomycete replicon fragment, the E. coli plasmid pBR322, and genes conferring resistance to neomycin and thiostrepton in streptomycetes.

The SCP2* replicon was further delineated by introducing a series of deletions into the shuttle plasmid pHJL192 by digestion with the restriction enzyme KpnI. Ligation, transformation into Streptomyces griseofuscus C581 (ATCC23916) and selection for neomycin resistance identified a functional replicon on a 4.6 kb EcoRI-KpnI restriction fragment of SCP2* that was contained in the shuttle plasmid pHJL201. Addition of a 1.0 kb BclI restriction fragment with the gene for thiostrepton resistance generated two shuttle vectors pHJL210 and pHJL211 that are genetically similar to pHJL197 and pHJL198. The amount of pHJL210 is approximately ten fold greater than the amount of pHJL197 when plasmid DNA's are isolated from cultures of S. griseofuscus. Therefore, the 1.3 kb KpnI-SalI deletion disrupts control of plasmid copy number.

Utility of the moderately high copy number vectors was tested by constructing genomic DNA libraries of S. fradiae T774 (a mutant of ATCC19609) and Actinoplanes utahensis (NRRL12052). Direct construction of a genomic DNA library was unsuccessful with DNA isolates from S. fradiae because the frequencies of recombinants were too low. A genomic DNA library of S. fradiae is available in the vector bacteriophage lambda Charon 4 (poster by S.E. Fishman*, P.R. Rosteck, Jr., and C.L. Hershberger). S. fradiae DNA was successfully subcloned from the genomic DNA library in bacteriophage lambda Charon 4 into pHJL210; therefore, genomic DNA libraries of S. fradiae were constructed in S. griseofuscus. Genomic DNA libraries of A. utahensis were constructed in S. griseofuscus with pHJL210 as the vector. The genomic DNA libraries in S. griseofuscus were evaluated with screens for resistance.

1078467

212 Direct Repeat Structure Associated with DNA Amplification in *Streptomyces fradiae*. S. E. Fishman* and C. L. Hershberger, Eli Lilly and Company, Indianapolis, Indiana 46285.

We have previously identified a 10.5 kb DNA sequence which is highly amplified and tandemly repeated in the genome of a mutant, *Streptomyces fradiae* JS85. A genomic library of DNA from the parent of JS85 (*S. fradiae* T774) before amplification was screened by plaque hybridization to identify phage clones containing the unamplified 10.5 kb DNA sequence. Phage isolates were identified which contained DNA homology to the amplified DNA sequence. Restriction digestions and Southern hybridization were used to verify that the DNA structure of the phage recombinants were the same as in the parental strain. A composite restriction map of the amplifiable unit of DNA plus adjacent chromosome was constructed. An EcoRI-BamHI DNA sequence was used as a probe for analyses by Southern hybridization. The probe exhibited homology to both ends of the 10.5 kb DNA sequence. Detailed restriction mapping identified a 2.2 kb direct repeat at the ends of the amplifiable unit of DNA. A minimum of four copies of the 2.2 kb direct repeat sequence have been identified in *S. fradiae* T774.

213 A New Plasmid (pY033) from Streptomyces orientalis, A Vancomycin Producer. Y.K. OH*, L. FARE, D.P. TAYLOR and L.J. NISBET. Smith Kline & French Laboratories, Philadelphia, Pa. 19101.

S. orientalis NRRL 2452 produces the glycopeptide antibiotic, vancomycin. An arsenate-resistant mutant (V33) isolated at 10mM Na'Arsenate produced more vancomycin than the parent strain and was found to carry a large high copy number plasmid DNA named pY033. The plasmid, 33.5 Kilo bases, had a number of sites of restriction endonuclease cleavage, including a unique EcoRI site. A restriction endonuclease cleavage map was contructed. Further work is in progress to investigate the role of, and potential for, this plasmid in modifying vancomycin production.

1078468

214 A MECHANISM FOR THE SITE-SPECIFIC EXCISION AND INTEGRATION OF THE SLP1 ELEMENT OF STREPTOMYCES COELICOLOR: AND IMPLICATIONS FOR THE FORMATION OF AUTONOMOUS SLP1 PLASMIDS

Charles A. Omer and Stanley N. Cohen
Department of Genetics
Stanford University Medical School
Stanford, CA 94305

SLP1 is a genetic element in the *Streptomyces coelicolor* A3(2) chromosome which can be transferred to the closely related strain *Streptomyces lividans*, 66 where it exists as either a chromosomally integrated element or as an autonomous plasmid of from 10-14 kb(1). We have demonstrated that the integrated form of SLP1 ($SLP1^{int}$) is a 17 kb DNA element that appears to excise from tne *S. coelicolor* chromosome and exist transiently as a plasmid during transfer to a *S. lividans* $SLPI^{-}$ strain. This transiently-existing plasmid can direct its site-specific integration into the *S. lividans* chromosome at a unique locus that corresponds to the original chromosomal location of $SLP1^{int}$ in *S. coelicolor*. Plasmids that contain the complete 17 kb SLP1 sequence were constructed, 1) by *in vitro* circularization of restriction endonuclease generated DNA fragments of chromosomal DNA carrying a tandem duplication of $SLP1^{int}$; 2) by molecularly cloning in the *E. coli* plasmid pACYC177 of a 17 kb *Bam*HI restriction fragment that contains the complete $SLP1^{int}$ sequence. When these plasmids were used to transform *S. lividans* $SLP1^{-}$, the resulting transformants contained the SLP1 sequence chromosomally integrated at the same site as when the $SLP1^{int}$ sequence is transferred by mating.

The physically autonomous SLP1 plasmids obtained by mating *S. coelicolor* with *S. lividans* are structural subsets of the transiently existing 17 kb

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complete SLP1 plasmid. The largest SLP1 plasmid isolated by matings, SLP1.2, retains the 1.72 kb Pst I fragment that contains the attachment site for SLP1; but has deleted another region required for integration. The smaller SLP1 plasmids have lost both the attachment site and the region deleted in SLP1.2. We have constructed an SLP1 plasmid in which only the 1.72 kb Pst I fragment which contains the attachment site and an adjacent 0.4 kb Pst I fragment are deleted. This constructed plasmid also fails to integrate into the chromosome.

A model for the site-specific excision and integration of $SLP1^{int}$ and a possible mechanism for the formation of the autonomous SLP1 plasmids will be presented.

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1078470

215 DNA reiterations in Streptomyces glaucescens: cloning and homology analysis of independently isolated sequences

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Mamoru Hasegawa*, Jacqueline Piret, Gilberto Hintermann, and Ralf Hütter

Mikrobiologisches Institut ETH, CH-8092 Zürich, Switzerland

*Kyowa Hakko Kogyo Co. LTD, Tokyo Research Laboratories, Tokyo, Japan

A widespread phenomenon among Streptomyces is the occurrence at unusually high frequencies of certain mutations affecting the morphological or biochemical behavior of the strain. One form of this genetic instability can be observed as tandemly reiterated DNA of specific chromosomal regions. Ten independently isolated reiterated sequences from S.glaucescens were cloned and analysed by restriction digests and hybridisations. The amplified sequencies occupied up to 45% of the total genomic DNA and ranged in size from 2.9-35 Kb per single unit. Cross-hybridisation between the cloned sequences and hybridisations to total DNA of the wild type DNA of S.glaucescens revealed that some reiterations arise from single copy, others from low copy amplified sequences. All reiterated sequences cloned could be allocated to two regions of the S.glaucescens genome. Furthermore some amplifications were accompained by adjacent deletions.

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16 Structural and functional characterisation of two unstable genes of *Streptomyces glaucescens*

Gilberto Hintermann and Ralf Hütter

Mikrobiologisches Institut ETH, CH-8092 Zürich, Switzerland

Streptomyces glaucescens produces on a suitable medium the black pigment melanin. The enzyme responsible is a tyrosinase. The gene responsible for tyrosinase production was cloned and used for the characterisation of melanin-negative mutants. Mutations affecting tyrosinase production were identified as either structural gene deletions or as mutations involved in the regulation of the expression of the intact structural gene.

Another feature of *Streptomyces glaucescens* is the production of the aminoglycoside antibiotic hydroxystreptomycin. The gene conferring resistance to streptomycin-like antibiotics was cloned. High frequency mutations leading to streptomycin-sensitivity and streptomycin non-production were identified as large genomic deletions comprising the structural gene for a 6-hydroxystreptomycin phosphotransferase and its flanking sequences.

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217 IS110: an insertion element of Streptomyces coelicolor

K. F. CHATER, C. J. BRUTON, S. G. FOSTER* and I. TOBEK**

John Innes Institute, Norwich, U.K.

Present address: *Glaxochem Ltd., Ulverston, Cumbria, U.K.

**Czechoslovak Academy of Sciences, Institute of Microbiology, Prague, Czechoslovakia

After propagation of attachment site-deleted derivatives of ϕC31 on Streptomyces coelicolor A3(2), three independently-obtained phages were found that could integrate into the S. coelicolor chromosome. They all contained apparently identical 1.6 kb insertions at the same position and in the same orientation in their DNA. This 1.6 kb sequence (IS110) was present in several copies in the S. coelicolor genome and absent from the closely related species S. lividans 66. Closely-related, but not identical, sequences were present in about 20% of a range of other Streptomyces species. The presence of IS110 in att-deleted ϕC31 phages containing a viomycin-resistance marker allowed the detection of S. coelicolor transductants containing the prophage integrated at the various IS110 copies. Linkage mapping of viomycin resistance in these strains has shown that at least two IS110 copies have well-defined chromosomal locations.

1078473

218 ISOLATION AND FUNCTIONAL ANALYSIS OF GENES INVOLVED IN MORPHOLOGICAL DIFFERENTIATION IN *STREPTOMYCES COELICOLOR* A(3)2

Jacqueline Piret* and Keith Chater**

*Mikrobiologisches Institut, ETH, Zürich, Switzerland
**John Innes Institute, Norwich, England

The morphological differentiation of *Streptomyces coelicolor* colonies is an apparently complex process whereby vegetative substrate mycelium gives rise to aerial branches which in turn develop chains of spores. Concurrently numerous secondary products are made. *S. coelicolor* A(3)2 mutants unable to produce aerial hyphae form colonies which look bald (*bld*). There are four known classes of *bld* mutants. Using a ØC31 phage vector, we have isolated two DNA sequences which restore the capacity to sporulate to two different classes of *bld* mutants: *bldA* and *bldB*. The phage derivatives carrying the cloned sequences permitted the construction of partial diploids for the *bld* regions by virtue of phage integration and establishment of lysogeny in the host chromosome. Functional analysis of such mutant lysogens indicated that the *bldA* region comprises at least two genes, one of which is terminally deleted in the cloned insert. Subcloning experiments placed the *bldA* genes at one end of the cloned DNA, supporting the idea that part of the *bldA* region is missing. A small fraction of mutant lysogens remain bald. Such colonies release phages which carry *bld* mutations rather than wildtype sequences and which apparently arise through some mechanism of homogenotization. Phages carrying *bldA* mutations could be used to show that *bldA* genes had indeed been cloned and to perform genetic complementation tests and fine structure mapping of *bldA* mutants.

1078474

219 Molecular biology of a streptomycete operon for glycerol utilisation

C. P. SMITH and K. F. CHATER

John Innes Institute, Norwich, U.K.

Physiological, genetical and gene disruption studies have shown that glycerol catabolism in Streptomyces coelicolor is determined by an operon (gyl) which is subject to substrate induction and catabolite repression (Seno and Chater, J. Gen. Microbiol. 129, 1403 (1983); Seno et al., Mol. Gen. Genet. 193, 119 (1984)).

Molecular cloning of the entire gyl region and subsequent S1 nuclease mapping and "Northern" blotting of gyl mRNA have shown that the operon spans 5.4 kb, and contains two transcription termination sites separated by 1.1 kb. Sequences normally essential for glycerol catabolism do not extend beyond the first termination site. These studies also revealed a 0.9 kb mRNA species immediately upstream of the gyl operon, which may encode a gyl regulatory protein.

DNA sequencing of a 500 bp region containing the 3' end of the "regulatory" gene, the gyl promoter, and the 5' end of the gyl structural genes, has revealed terminator-like structures, a ribosome binding site and open reading frames that correlate well with the predicted organisation of the gyl operon.

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220 Cloning and Preliminary Characterization of a Tylosin-Resistance Gene from A Tylosin-Producing Strain of Streptomyces fradiae. V.A. Birmingham, K.L. Cox, S.E. Fishman, P.R. Rosteck, Jr., C.L. Hershberger, and E.T. Seno, Eli Lilly and Company, Indianapolis, Indiana 46285.

A DNA fragment, which confers resistance to high tylosin concentrations in Streptomyces lividans and Streptomyces griseofuscus, was cloned from a tylosin-producing strain of Streptomyces fradiae, T776. The tyl^R clone was obtained by partial Sau3A digestion of S. fradiae DNA, ligation into the BglII site of the high-copy plasmid vector, pIJ702, and transformation into S. lividans TK23. Screening for tylosin resistance (500 μg/ml) among the thiostrepton-resistant transformants led to the identification of eleven recombinant plasmids capable of transforming S. lividans to tylosin resistance. Restriction enzyme analysis of five of the tyl^R plasmids revealed a common 2.6 Kb KpnI fragment, which alone could cause tylosin resistance in S. lividans when present in either orientation in the KpnI site of pIJ702.

PSVB2, one of the tyl^R plasmids containing a 4.9 Kb Sau3A insert, was further studied. It conferred resistance to several macrolide antibiotics (tylosin, niddamycin, carbomycin), lincomycin and vernamycin B in S. griseofuscus, a pattern associated with the MLS-resistance phenotype. PSVB2 failed to completely restore tylosin resistance in three tylosin-sensitive (Tyl^S) mutants of S. fradiae. Two of the mutants, JS85 and JS87, were obtained by regenerating protoplasts of Tyl^R strains. JS87 contains a known deletion encompassing aud, a sequence tandemly reiterated about 500 times in JS85 and present as a single copy in the parental strains. Southern hybridization, using pSVB2 as a probe, revealed that the cloned gene was present on the same restriction fragment in JS85, JS87 and other Tyl^S and Tyl^R strains of S. fradiae. Thus, the deletion associated with loss of tylosin production and tylosin resistance in JS87 did not include the cloned tyl^R gene.

The data suggest that the cloned S. fradiae tyl^R gene is an mls resistance gene, or a gene activating expression of an endogenous mls determinant, and that the cloned gene is poorly expressed in some Tyl^S mutants of S. fradiae.

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221 Cloning and analysis of the agarase gene of Streptomyces coelicolor A3(2)

R. JOSEPH*, G. H. JONES+, J. M. WARD and M. J. BIBB

John Innes Institute, Norwich, U.K.

Present address: *CFTRI, Mysore - 570013, India. +University of Michigan, Division of Biological Sciences, Ann Arbor, Michigan 48109, U.S.A.

Streptomyces strains produce a variety of extracellular proteins that permit the breakdown and utilisation of extracellular macromolecules. An unusual example is agarase secreted by S. coelicolor A3(2). This enzyme degrades agar into small oligosaccharides that can ultimately be absorbed into the cell and utilised as sole carbon source. The agarase gene is subject to carbon catabolite repression and preliminary studies indicate that it is also inducible by the breakdown products of agarase. The gene resides in the 9 o'clock region of the linkage map and contains, or lies close to, a site for integration of the plasmid SCP1 (the resulting NF strains fail to produce agarase). In order to study the regulation of expression of the agarase gene and the process of secretion itself, the gene has been cloned from genomic DNA of S. coelicolor A3(2) into S. lividans TK24, employing the vectors pIJ61 and pIJ702. These clones and some of those obtained from subsequent subcloning experiments appear to respond normally to catabolite repression, strongly suggesting that the regulatory sequences of the gene have also been isolated. The coding sequence of the agarase gene has been limited to a region of about 1.8 kb. Examination of the culture broths obtained from the growth of several clones indicates that, in some, agarase constitutes over half of the total extracellular protein. The intracellular and extracellular forms of the protein have been partially purified and subjected to SDS-PAGE. The extracellular enzyme appears to be about 28-30 kb in size and that of the intracellular form slightly larger, suggesting the existence of a leader peptide analagous to those of other secreted proteins.

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222 The ribosomal RNA genes of *Streptomyces coelicolor* A3(2)

H. A. BAYLIS and M. J. BIBB

John Innes Institute, Norwich, U.K.

The 23S, 16S and 5S rRNAs of *S. coelicolor* were purified from low melting point agarose and, after labelling with polynucleotide kinase and ^{32}P, were used individually as hybridisation probes against genomic DNA of *S. coelicolor* A3(2) that had been cleaved with a variety of restriction enzymes. Six sets of rRNA genes were detected. A mixture of 16S and 23S rRNA probes was also used to analyse genomic DNA of *S. lividans* 66 and *Escherichia coli*. *S. lividans* gave an identical hybridisation pattern to that of *S. coelicolor* and the rRNA operons of *E. coli* could also be clearly identified, giving a characteristic but much weaker hybridisation pattern.

Screening of a *S. coelicolor* genomic library in phage λ EMBL4 using ^{32}P labelled rRNA has resulted in the identification of clones carrying rRNA genes. Subsequent restriction analysis and Southern hybridisation of the phage DNAs with ^{32}P labelled *S. coelicolor* rRNA has shown that at least four of the six sets have been isolated. One of these clones, λ RSC33, has been analysed further and the order of the genes on it determined by Southern hybridisation using purified r RNAs as: 16S - 23S - 5S. The genes are closely linked; all three are contained in a maximum of 5.5 kilobases of DNA.

Preliminary results using ^{32}P labelled tRNA hybridised against either genomic DNA or λ RSC33 DNA suggests that tRNA genes may be closely associated with the rRNA genes. The promoter regions and the chromosomal map locations of these genes are currently under study.

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223

Characterization and chromosomal mapping of chloramphenicol non-producing mutants of *Streptomyces venezuelae*.

J. Doull, C. Stuttard*, & L.C. Vining[1]
Departments of Microbiology, and [1]Biology
Dalhousie University, Halifax, N.S., CANADA

Mutagenesis with UV(254nm), EMS, or 8-MOP-NUV, gave twelve independent mutants of *Streptomyces venezuelae* unable to synthesize chloramphenicol. Mutants Cml-1 and Cml-12 were able to convert chorismic acid to *p*-aminophenylalanine, but produced chloramphenicol when supplied with *p*-aminophenylalanine. Mutants Cml-4, Cml-5 and Cml-8 accumulated *p*-aminophenylalanine and were probably blocked in the hydroxylation of *p*-aminophenylalanine to *p*-aminophenylserine. Cml-2 accumulated three non-chlorinated analogues of chloramphenicol indicating that chlorination of the α-N-acyl group was blocked. The remaining six mutants did not excrete any detectable direct or modified precursor of chloramphenicol.

Conjugational crosses for initial chromosomal gene mapping were done with a fertile, streptomycin-resistant (*strA*) derivative (VS206) of a relatively high-level chloramphenicol-producing strain, VS191 (*his-6* *ade-10*), a protoplast regenerant of strain VS113 identified in patch tests for lethal zygosis and fertility. Strain VS206 was crossed with singly auxotrophic derivatives of Cml-1, Cml-5, and Cml-7. In each cross the *cml* mutation consistently mapped to the chromosome between *his-6* and *strA6*. The other nine *cml* mutations were mapped to the same location using prototrophic, Sm^S Cml^- mutants in crosses with VS206. This evidence for chromosomally-located structural genes controlling chloramphenicol biosynthesis in *S. venezuelae* is consistent with our earlier evidence against involvement of detectable plasmid in this pathway. Also, strain VS191 and a further Ltz-sensitive derivative, VS194, synthesized chloramphenicol in amounts similar to the parental strain VS113, suggesting that presumptive loss of one or two physically undetected fertility plasmids likewise had no effect on chloramphenicol biosynthesis.

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224 Exocellular Esterase Production by Phytopathogenic *Streptomyces*

Janet L. Schottel[1], Leann Canty[2] and Ross McQueen[2]
Department of Biochemistry[1] and Department of Plant Pathology[2]
University of Minnesota, St. Paul, MN 55108

Streptomyces scabies is a gram positive soil bacterium that is pathogenic on potato tubers. Penetration of the protective covering on underground plant parts, composed of the polyester compound suberin, may be a necessary prerequisite to invasion by this organism. Cutin is a similar type of polyester compound that covers the aerial parts of plants. Esterases that are secreted into the growth medium may be related to the penetration of these polyesters by the bacterium. We have isolated nine different strains of *S. scabies* from scab lesions on potato tubers and have tested them for their ability to produce exocellular esterases.

The *Streptomyces* isolates were grown on a variety of different compounds as carbon sources to test for specific induction of esterases. These carbon sources included glucose, glycerol, chitin, cellulose, docosane, octadecane, n-nonocosane, lauric acid myristyl ester, palmitic acid methyl ester and cutin. The substrates used for the enzyme assay were p-nitrophenylbutyrate (PNPB) and p-nitrophenylpalmitate (PNPP). We found that four of the isolates produced an exocellular esterase that utilized either PNPB or PNPP as a substrate; two of the isolates were able to utilize both compounds as substrates. Organisms grown on cutin as a sole carbon source produced five to ten times more esterase then organisms grown on the other carbon sources. No detectable esterase activity was observed when the bacteria were grown with glucose or glycerol as the sole carbon source. The exocellular production of esterase was repressed when the organisms were grown on cutin plus glucose; cAMP added to the culture was found to prevent the repression by glucose.

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225 Use of the E. coli galactokinase gene for the isolation and characterization of Streptomyces promoter sequences

Mary E. Brawner*, Jeffrey I. Auerbach, Martin Rosenberg, and Dean P. Taylor
Smith Kline & French Laboratories, 1500 Spring Garden Street, Philadelphia, PA 19101

Streptomyces are gram positive soil bacteria which undergo a complex developmental cycle and are responsible for the production of the majority of the clinically significant antibiotics. Antibiotic production and the developmental cycle are genetically regulated processes that are attractive model systems for the study of gene regulation in the actinomycetes. Unfortunately, many of the genes involved in antibiotic biosynthesis have not been characterized and their gene products are not easily detected.

In procaryotes and eucaryotes, promoter sequences which regulate genes whose products are not readily assayable have been characterized using transcriptional fusions. The fusions can be constructed in vitro by inserting the promoter sequence at a site upstream of a gene which encodes an assayable product in plasmids referred to as promoter probe vectors. We have developed Streptomyces promoter probe plasmids which contain the E. coli galK gene. The plasmids are E. coli-Streptomyces shuttle vectors which permits the rapid assembly and characterization of the plasmid constructions in E. coli followed by transformation into Streptomyces for functional analysis. The galK gene is preceded by a series of unique cloning sites and a fragment, containing the tR_1 transcription terminator of coliphage lambda, which prevents readthrough transcription into galK. To test the utility of the promoter probe vector fragments thought to contain Streptomyces promoter sequences were inserted into the cloning sites preceding galK. One of the fragments was from the B-gal gene system of S. lividans and a second fragment contained the promoter from the S. fradiae aph gene. Both fragments stimulated transcription of the galK gene when inserted in the correct orientation but did not promote galK expression in the opposite orientation. The galactokinase produced in S. lividans from the E. coli gene had the same electrophoretic mobility as galactokinase purified from E. coli and can be detected enzymatically and immunologically.

S. lividans naturally produces a galactokinase necessitating the isolation of a galactokinase deficient derivative for these studies. A galactokinase mutant of S. lividans was isolated by UV mutagenesis of a spore suspension and selected on 2-deoxygalactose to inhibit the growth of cells that produce galactokinase. The mution in S. lividans 1326/12K$^-$ is complemented by expression of the E. coli galK gene. Complementation is observed on minimal media containing galactose as the sole carbon source or on a pH indicator plate which can distinguish gal$^+$ versus gal$^-$ cells. Positive and negative selection for expression of galactokinase in E. coli require complementation of the host galactokinase mutation by expression of galK from pKO plasmids. These selection systems have proven useful for studying promoter sequences cloned into the pKO promoter probe system. The potential of such positive and negative selections and the pH indicator medium for the isolation and manipulation of Streptomyces genetic regulatory elements will be presented.

226 Isolation and Characterization of a New Actinophage (øSE1) for Streptomyces erythreus. A. D. GRUND* and C. R. HUTCHINSON. School of Pharmacy, University of Wisconsin, Madison 53706.

A new actinophage, øSE1, was isolated from soil on Streptomyces erythreus NRRL 2338, a strain that is resistant to infection by several of the well-known actinophages. It exhibited lytic growth on eight strains of S. erythreus and temperate growth on two other strains, but did not infect 31 other species of Streptomyces. Plaque formation was stimulated by the presence of Ca^{2+} and Mg^{2+} ions. Electron microscopy revealed the intact phage virions to have a hexagonal head outline (55 nm width), ringed tail (130 nm length), and three or four short tail fibers. The phage genome was found to be linear, double-stranded DNA with a size of ca. 41.5 kb as determined by electron microscopy. Data from agarose gels indicated three or fewer cutting sites for several restriction endonucleases including EcoRI, BglII, ClaI, HindIII and StuI, and provided a rudimentary genomic physical map. No evidence for cohesive ends was found by two different methods. These and other results suggest a headful packaging mechanism for øSE1. Studies of its natural variants, deletion mutants constructed in vitro, and genetic properties are in progress with anticipation that this new actinophage will be useful in studies of the genetics and biochemistry of macrolide antibiotic formation in S. erythreus strains.

227 A Model System for Studying the Genetics and Biochemistry of Anthracycline Antibiotic Formation. Mutants of Tetracenomycin C Biosynthesis in Streptomyces glaucescens. H. MOTAMEDI, E. WENDT-PIENKOWSKI, and C.R. HUTCHINSON. School of Pharmacy, University of Wisconsin, Madison, WI 53706.

Streptomyces glaucescens GLA.0, which produces tetracenomycin C (TCM C) an anthracycline antibiotic with antitumor properties, was treated with NTG, UV and DES singly as well as in pairwise combinations and the surviving colonies screened by bioassay against S. coelicolor M111. Stable, non-producing (tcm) mutants resulted at an average frequency of 0.1% (single treatment). Thin layer chromatography of EtOAc extracts of these mutants grown in liquid culture revealed the accumulation of several putative intermediates of the TCM C biosynthetic pathway, as well as new compounds not present in the wild-type strain. Mutants with no apparent accumulation of TCM C biosynthetic intermediates also were found. The mutants had wild-type growth, sporulation and TCM C resistance properties. Classification of these mutants into secretor-convertor groups using cosynthesis and precursor feeding experiments is being studied.

Protoplasts of the GLA.0 strain can be transformed efficiently with two Streptomyces plasmid cloning vectors, pIJ702 and pIJ922. These vectors and others under investigation will be used with the tcm mutant strains in future cloning and functional studies of the genes governing the biosynthesis of TCM C.

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228 Mutation, recombination, and gene mapping in the erythromycin producing bacterium, Streptomyces erythreus NRRL 2338. J.M. Weber,* C.K. Wierman, and C.R. Hutchinson. University of Wisconsin, Madison, WI

Streptomyces erythreus NRRL 2338 produces erythromycin A, a macrolide antibiotic of longstanding medical importance since its discovery in 1952. In addition to the industrial significance of genetic research in this organism, important biological questions may also be addressed relating to the organization and developmental control of the expression of antibiotic biosynthetic genes. This is a report of the first genetic study in S. erythreus NRRL 2338 and the development of a genetic system in this organism to study erythromycin biosynthesis.

Mutant strains of S. erythreus unable to produce erythromycin were isolated following ultra-violet light and chemical mutagenic treatments. These mutants were found to be blocked at four different stages in erythromycin biosynthesis based on results from antibiotic co-synthesis assays, identification of erythromycin pathway intermediates in growth media, and biotransformation of pathway intermediates by the mutants. Twenty types of auxotrophic mutations, four types of drug-resistance mutations, and two types of morphological mutations were also found.

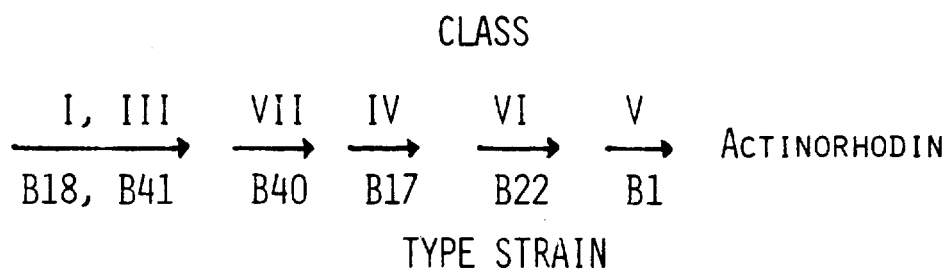
A natural form of genetic recombination was demonstrated in S. erythreus through conjugal mating experiments in which stable prototrophic progeny were isolated from mixed cultures of auxotrophic parent strains. Quantitative data on recombination were then obtained through analysis of selected haploid recombinants from four and five-factor crosses. A genetic map including thirteen mutations arranged in a circular sequence was produced from the combined results of these crosses. Two mutations that blocked erythromycin biosynthesis in formation of the erythronolide macrolactone mapped to the same interval on the chromosome. Other preliminary results suggested that these and other mutations in macrolactone synthesis are located very close to one another. Mutations affecting biosynthesis of the erythromycin sugars also appeared to be located on the chromosome, but are probably not close to the mutations in macrolactone synthesis.

1078483

229 CHEMICAL AND GENETIC ANALYSIS OF ACTINORHODIN BIOSYNTHESIS IN *Streptomyces coelicolor*

Sheri P. Cole, Xian-Guo He, Brian A. M. Rudd, Paul J. Keller, Ching-jer Chang and Heinz G. Floss

Streptomyces species produce about two-thirds of the world's antibiotics, yet little work has been done on the genetics of the producing organism, especially with respect to biosynthesis. *Streptomyces coelicolor* is the genetically best characterized actinomycete, with over one hundred genes mapped to its circular chromosome. The genes controlling the biosynthesis of actinorhodin (1, figure 3), an acid-base indicating antibiotic, have been mapped to a short segment on this chromosome. A large number of mutants defective in actinorhodin biosynthesis were isolated and grouped into seven classes by Rudd and Hopwood (1). The classification was based on each mutant's ability to secrete or convert an intermediate in the biosynthesis to actinorhodin, as shown below:



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Two classes which secrete intermediates were studied (IV and V), and the results of these investigations are the subject of this abstract.

The structure of an unstable, yellow intermediate produced by B1, a class V mutant, has been determined. Comparison of its UV-visible spectrum with related antibiotics (2), suggests the presence of an oxidized naphthalene skeleton. The IR of this intermediate gives evidence for conjugated carbonyl (1643 cm^{-1}), hydroxyl (3433 cm^{-1}) and carboxylic acid (1716 cm^{-1}) groups. The bulk of the data came from ^{1}HNMR experiments, some of which are summarized in figure 1. Single frequency decouplings of the resonances at 7.397, 6.747, 6.788 and 6.270 ppm were used to establish the connectivities of these protons. Additional decoupling experiments at 4.761ppm and at 6.270 ppm gave information on the structure and point of attachment of the aliphatic portion of the molecule.

The other compound we have isolated is produced by B40, a class IV mutant which is the first secreting strain of the sequence. This compound is not an active intermediate in the biosynthesis, but rather a shunt product which is relatively more stable. Like actinorhodin, it is produced from eight acetate units, but the cyclizations have occurred at different positions on the polyketide chain. The IR spectrum indicates a carbonyl stretching band at 1678 cm^{-1} and a carboxylic acid O-H stretch at 3095 cm^{-1}. ^{1}HNMR and ^{13}CNMR data are summarized in figure 2. The point of attachment of the side chain to the aromatic portion of the molecule cannot be determined with certainty from the above techniques of analysis, and awaits X-ray diffraction for final clarification.

A partial scheme can be drawn for actinorhodin biosynthesis, as shown in figure 3. Much work remains to be done concerning the intermediate steps of

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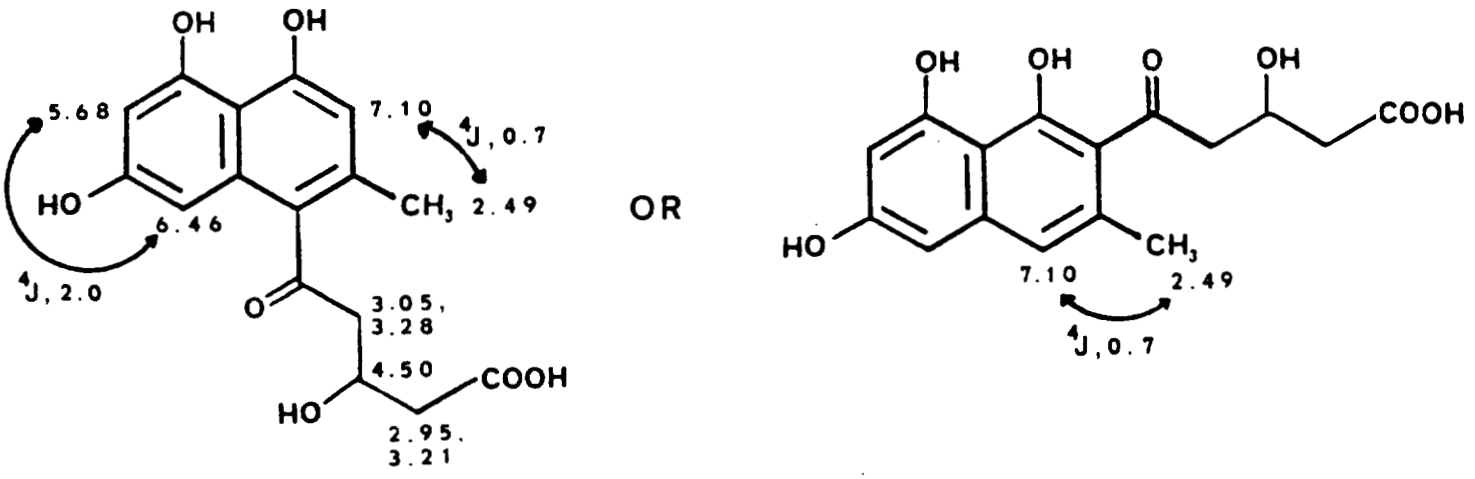
Figure 2. Some NMR data of the B40 shunt product. A. ^{1}HNMR in DMSO-d_6, B. ^{13}CNMR in CD_3OD.

A



B

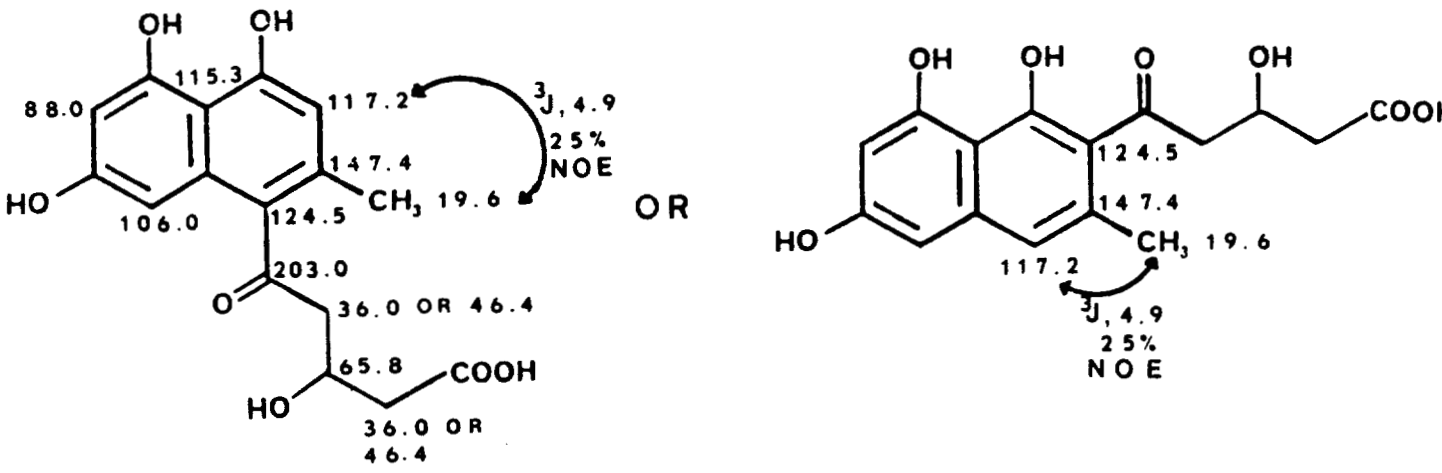


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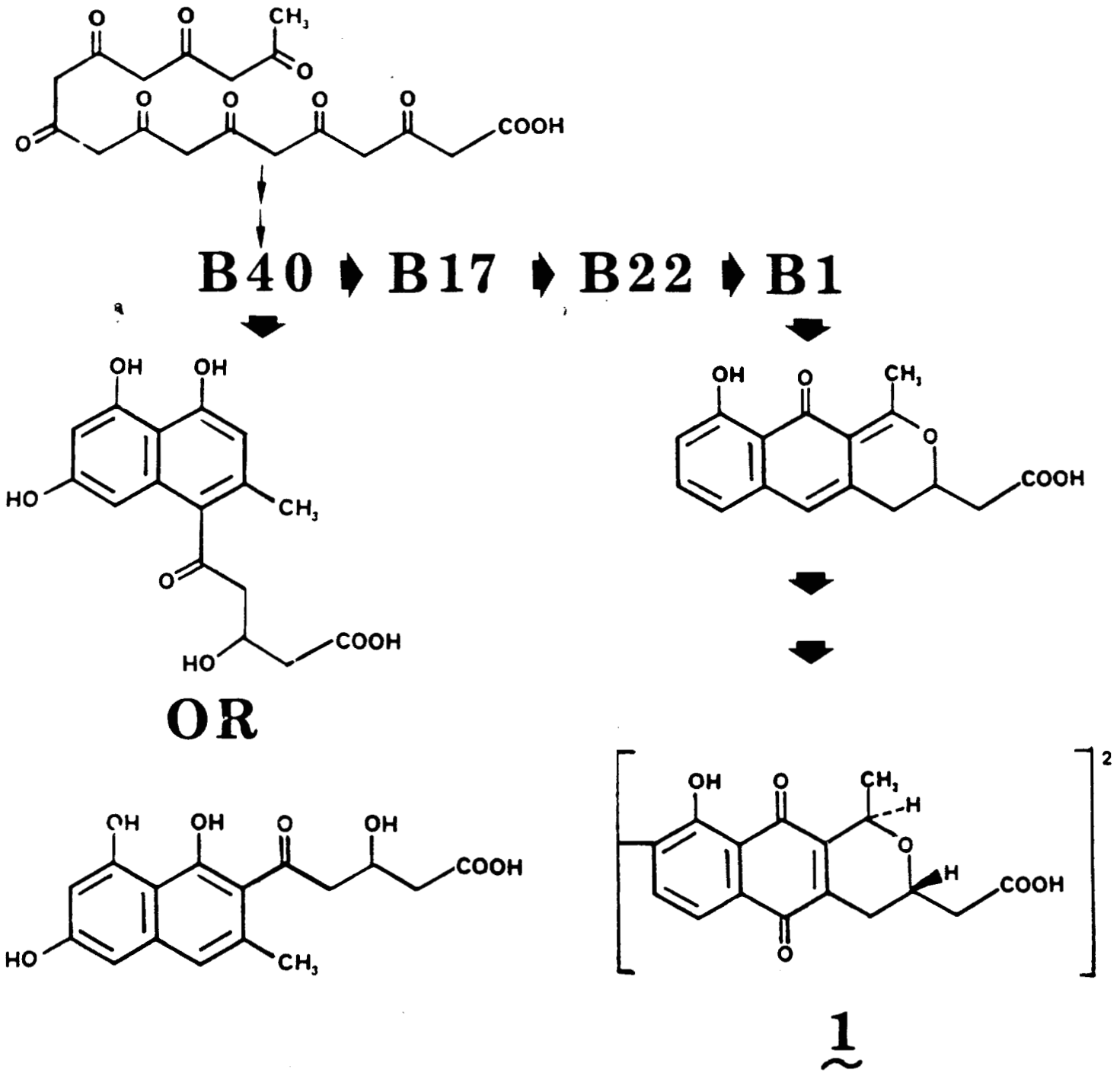
Figure 3. Biosynthetic scheme for actinorhodin.



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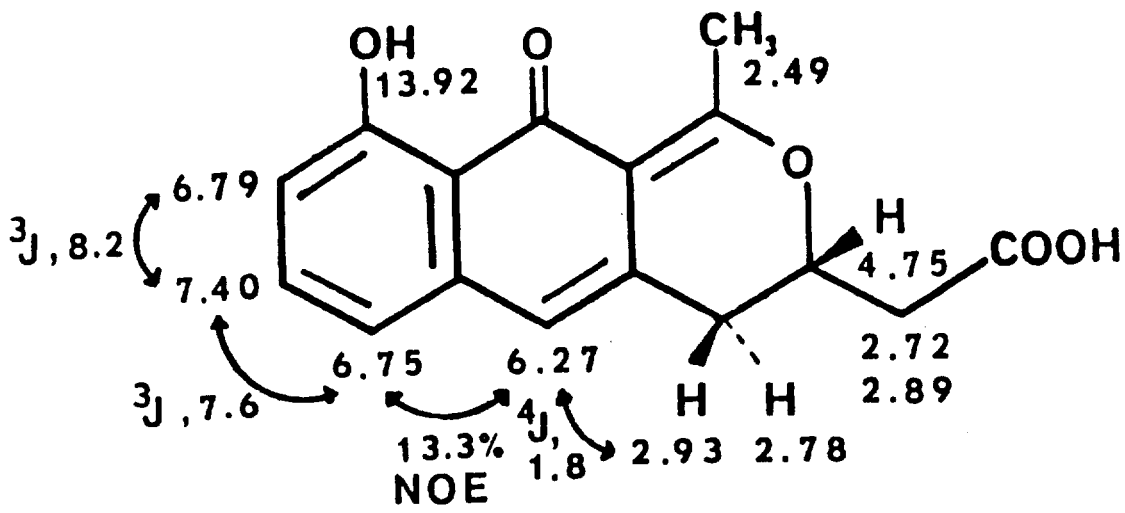
actinorhodin biosynthesis, and with the aid of blocked mutants, elucidation of these intermediate structures may be possible.

Figure 1. Some ^{1}HNMR data of the B1 intermediate in $CDCl_3$



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Abstract for Bloomington ASM Meeting

J. S. Feitelson

Medical Research Division, American Cyanamid Company
Lederle Laboratories, Pearl River, NY 10965

The production of the red pigmented antibiotic produced by Streptomyces coelicolor A3(2), undecylprodigiosin, was studied using molecular genetic and biochemical techniques. Over 21 Kb of genomic DNA was cloned which "complemented" several specific mutations in the red gene cluster. Subcloning experiments allowed the physical mapping of each red gene (redA, B, E, and F) to different regions within the cloned segments. Mutational cloning in the actinophage ØC31 suggested that there are at least two transcription units in the red region.

Biochemical studies using sonicated extracts or gently protoplasted cells indicated that there were two forms of O-methyltransferase activity on gel filtration columns: a very high molecular weight peak ($>5x10^6$ d) and a 49 Kd peak. Analysis of extracts from red mutants were consistent with the idea that these two forms are related, and that at least both redE and redF gene products are necessary for functional O-methyltransferase activity in vivo. Lack of functional activity of the redE gene in a heterologous host, S. glaucescens, provided additional support for the necessity of a biosynthetic complex involving several red gene products for efficient expression.

Fermentation experiments in liquid production media extended the observation originally made in Serratia marcescens that prodigiosin compounds are classic examples of "secondary" metabolites. The specific activity of O-methyltransferase rises after a lag with biomass and peaks at the onset of antibiotic production. Thus, understanding the mechanisms of genetic regulation of undecylprodigiosin biosynthesis in S. coelicolor may have relevance to other antibiotic production pathways, and perhaps to secondary metabolism in general.

1078489

231 Development of Streptomyces toyocaensis as a Gene Cloning System.

MICHAEL D. JONES AND JEFFREY T. FAYERMAN, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

Streptomyces toyocaensis, a producer of a glycopeptide like antibiotic, was examined for its potential as host organism in a gene cloning system. Parameters examined included the ease with which protoplasts could be obtained, the regeneration frequency of the protoplasts, and the ability of the protoplast to take up covalently closed circular DNA (cccDNA) by polyethylene glycol mediated transformation. Procedures for protoplast preparation, regeneration and transformation of S. toyocaensis differ from published methods for manipulating other Streptomyces species. It was determined that optimal protoplast regeneration is obtained when protoplasts are prepared at room temperature by lysozyme treatment of mycelia grown at 37 degrees in media containing 0.4 percent glycine. Protoplasts regenerate after approximately three days incubation at 30 degrees. Transformation by cccDNA is linear with respect to the concentration of DNA in a range of 5 ng to 5 μg per assay. The addition of calf thymus DNA had no appreciable effect on transformation although it has been shown to increase transformation frequency in some systems. Protamine sulfate, which increases transformation frequency in S. fradiae and S. ambofaciens, was detrimental to transformation of S. toyocaensis. Currently, under optimum conditions, 1×10^5 transformants per μg cccDNA are obtained using approximately 1×10^9 S. toyocaensis protoplasts.

232 Heterologous Promoter Recognition by Streptomyces ambofaciens.

MARK A. RICHARDSON, RAMAMOORTHY BELAGAJE and JEFFREY T. FAYERMAN, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

Plasmids have been constructed using the Bacillus subtilis veg promoter to drive expression of heterologous genes in Streptomyces. Streptomyces ambofaciens transformants were selected for expression of either the Escherichia coli β-galactosidase or hygromycin phosphotransferase gene. Assays of hygromycin phosphotransferase activity indicated that expression driven by the veg promoter may be greatly enhanced during late stationary phase. A synthetic promoter, closely resembling the veg promoter, was prepared and was also shown to drive expression of the hygromycin phosphotransferase gene in S. ambofaciens. The synthetic promoter is contained on a 72 base pair EcoRI-BamHI fragment with an internal HindIII site separating the promoter and the ribosome binding site.

233 MUTANTS FOR MUTASYNTHESIS. J. Majer, Northwestern University Medical and Dental Schools, Chicago, IL 60611.

Streptomyces venezuelae ATCC 15068 is a versatile producer of macrolide monoglycosides including the 12-membered constituents of the methymycin complex and the 14-membered congeners of pikromycin. Colonies of the parent strain after treatment with N-methyl-N'-nitro-N-nitrosoguanidine were screened for non-producers. These were further separated into aglycone producers (Agl^+) and aglycone non-producers (Agl^-). The latter were then assayed for production of the sugar intermediates (Sug^+) and for the glucosyltransferase activity (Gte^-) in arrangements in which the aglycone, for example, narbonolide, was supplemented. The Agl^-, Gte^+, Sug^+ idiotrophs thus identified were examined for their ability to utilize selected mutasynthons from the macrolide and non-macrolide series.

234 PROTOPLAST TRANSFORMATION AND TRANSFECTION OF THE ANTHRACYCLINE PRODUCERS STREPTOMYCES PEUCETIUS AND STREPTOMYCES C5

Jay S. Lampel and William R. Strohl.

Department of Microbiology, The Ohio State University, 484 W. 12th Ave. Columbus, OH 43210.

The anthracycline-producing Streptomyces peucetius and Streptomyces C5 (over-producer of ε-rhodomycinone) were converted to protoplasts from vegetatively growing mycelia. Conditions are described for maximal protoplast formation (>99%) and for regeneration frequencies over 50%. Streptomycete plasmids pIJ 61, pIJ 361, and pIJ 702 were isolated from S. lividans 1326, and successfully introduced into S. peucetius and Streptomyces C5 by polyethylene glycol (PEG) assisted protoplast transformation. Frequencies of up to 10^{-} transformants per μg of plasmid DNA were achieved by these procedures. The plasmid DNA isolated from a few randomly selected transformants was analyzed by agarose gel electrophoresis. Restriction endonuclease digestion of these recovered plasmids indicated that the two anthracycline-producing strains can stably harbor the plasmids without deletion of plasmid sequences or loss of the plasmids for up to three transfers through selection media. S. peucetius and Streptomyces C5 mycelia were also sensitive to infection by actinophage ØC31 and its derivative KC401. Furthermore, protoplasts of these strains were transfected with ØC31 and KC401 DNA using PEG-assisted protoplast transfection. Optimal conditions were determined for transfection of the protoplasts.

10184191

235 ISOLATION AN PARTIAL CHARACTERIZATION OF A NEW TEMPERATE ACTINOPHAGE FROM THE ANTRACYCLINE PRODUCER STREPTOMYCES GALILAEUS ATCC 31133

Sabine P. Plocher, Jay S. Lampel, and William R. Strohl.

Department of Microbiology, The Ohio State University, 484 W. 12th Ave. Columbus, OH 43210.

A new temperate bacteriophage was isolated from Streptomyces galilaeus ATCC 31133 by crossing with various species of Streptomyces. Sensitive hosts became lysogenized and yielded turbid plaques of 2 to 3 mm in diameter. The bacteriophage can be propogated in liquid culture and on solid media by standard infection, with a pH optimum of 6.8 to 7.0. The phage is not efficiently induced by mitomycin C or UV irradiation. Host range analysis indicated that 16 of 28 species of Streptomyces tested were sensitive to infection on solid medium. Streptomyces lidivans and S. coelicolor A3(2) strains M110, M130, and M124 were among those not infected by this new actinophage. Purified phage particles were examined by electron microscopy. Morphologically this actinophage belongs to Group B of Bradley's classification. It has an icosahedral head and a long noncontractile tail with small terminal appendages. The genome length of the phage was estimated by restriction endonuclease digestion and agarose gel electrophoresis and was determined to be 29 to 31 kb. This phage was restricted by Sal I, Pvu II, and Alu I but in all cases yielded many restriction fragments. No sites were detected for BamH I, Hind III, Pst I, Bgl II, Xba I, and Bcl II.

1078492

DEVELOPMENT OF GENETIC MANIPULATION SYSTEMS FOR THERMOMONOSPORA

Kenneth A. Pidcock, Bland S. Montenecourt, and Jeffrey A. Sands
Department of Biology, Lehigh University, Bethlehem, PA. 18015

Thermophilic actinomycetes are of significant scientific and industrial interest as producers of thermostable hydrolytic enzymes and antibiotics. One particularly interesting genus is Thermomonospora, several species of which have been shown to produce an efficient cellulase complex. While some aspects of the molecular biology of thermophilic actimomycetes can be studied by gene cloning into E. coli and Streptomyces, the development of endogenous cloning and genetic manipulation systems will be required for optimum strain development.

Starting from procedures devised for Streptomyces, we have developed protocols for efficient protoplast production and regeneration for Thermomonospora fusca, routinely obtaining protoplast suspensions of $\sim 10^8$/ml and regeneration frequencies of 0.1%, similar to values reported for other actinomycetes. Protoplast fusion followed by genetic recombination has been demonstrated with auxotrophic and drug resistant mutants of Thm. fusca.

Procedures for transformation and transfection of protoplasts of Thm. fusca and another thermophilic actinomycete, Saccharomonospora viridis, are being developed using plasmids and phages of Streptomyces (obtained from D. Hopwood) and Sam. viridis. Additional plasmids and phages of thermophilic actinomycetes are being isolated from environmental samples. Screening of thermophilic actinomycete isolates for the presence of plasmids has been greatly facilitated by a procedure which we have developed that yields ccc DNA preparations free of chromosomal DNA without ultracentrifugation.

The development of procedures for protoplast production and regeneration, recombination following protoplast fusion, and transformation and transfection provides a foundation for the development of endogenous gene cloning systems in thermophilic actinomycetes.

1078493

237 Efficient Transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts by plasmid DNA.

PATTI MATSUSHIMA and RICHARD H. BALTZ, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

Conditions for efficient transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts by plasmid DNA have been identified. Transformation frequences were influenced by the temperatures for cell growth, protoplast formation and protoplast regeneration; by the growth phase of the cells before protoplasting; by the concentration of protoplasts in the assay; by sodium chloride or sodium acetate concentration and by the source and concentration of polyethylene glycol, the transformation inducing agent. Transformation frequences were enhanced about 10-fold by adding heterologous DNA and protamine sulfate to the transformation assay. Under optimum conditions, *S. fradiae* and *S. ambofaciens* protoplasts were transformed with plasmids pFJ105 and pIJ702 at frequencies of 1.5×10^6 to 1.5×10^7 transformants/μg DNA. *S. fradiae* protoplasts were transformed at a 100-fold lower efficiency with pFJ105 isolated from *S. ambofaciens* rather than *S. fradiae*, suggesting that *S. fradiae* possesses a restriction/modification system(s). Mutants of *S. fradiae*, partially defective in restriction and more efficiently transformable by three different plasmids have been isolated and partially characterized.

238 Development of Alkaline Phosphatase as a Model System for the Analysis of Gene Expression in *Streptomyces*.

P. SOLENBERG, P. MOONEY and N. A. SCHAUS, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

To study the regulation of gene expression in *Streptomyces*, we have developed alkaline phosphatase as a model system. Analysis of the regulation of alkaline phosphatase gene expression may provide information on the more general mechanisms utilized by *Streptomyces* to regulate gene expression. When *Streptomyces ambofaciens* 15154 is grown under conditions of phosphate limitation, two alkaline phosphatase activities can be detected which differ in their localization and time of appearance. One activity is produced during vegetative growth in a semi-synthetic medium and is found released into the growth medium. When cells are grown in a minimal salts medium a second activity is produced late in the growth cycle. This activity is found outside the cell but cell associated. Both alkaline phosphatase activities have been purified and their relatedness analyzed.

10784944

IDENTIFICATION OF TWO FORMS OF RNA POLYMERASE FROM STREPTOMYCES COELICOLOR THAT DIFFER IN THEIR PROMOTER RECOGNITION SPECIFICITY AND THEIR SIGMA SUBUNITS: Janet Westpheling, Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts

Streptomyces coelicolor contains at least two forms of RNA polymerase which are distinguishable by their ability to utilize representatives of different promoter classes from Bacillus subtilis. To examine promoter recognition specificity of RNA polymerase from S. coelicolor we employed two well characterized templates from B. subtilis known as veg and ctc. The veg promoter is utilized in B. subtilis by the predominant form of RNA polymerase in vegetative cells ($E\sigma^{55}$) and has an "E. coli like" recognition sequence. ctc has a novel promoter sequence and is utilized by a minor form of RNA polymerase ($E\sigma^{37}$). Using veg and ctc containing DNAs as templates, partially purified preparations of RNA polymerase from S. coelicolor were assayed by in vitro run off transcription. Two forms of enzyme were identified which correspond to the $E\sigma^{55}$ and $E\sigma^{37}$ forms of B. subtilis holoenzyme in their promoter recognition specificity. Further purification of these enzymes revealed that they have the same subunit composition of core RNA polymerase (beta, beta prime and alpha) characteristic of prokaryotic RNA polymerases. In addition to the core subunits, several minor bands were observed to copurify with the transcribing activities. In heterologous complementation experiments in which these gel purified polymerase associated proteins from S. coelicolor were tested for their ability to stimulate ctc and veg transcription in the presence of core RNA polymerase from B. subtilis in in vitro transcription assays, it was possible to identify sigma factors for veg and ctc transcription. veg transcription is directed by a protein of approximately 35,000 daltons ($E\sigma^{35}$) and ctc transcription is directed by a protein of approximately 41,000 daltons ($E\sigma^{41}$). We have used the veg and ctc transcribing activities to identify and characterize a Streptomyces promoter with a novel recognition sequence (Ranes and Westpheling, abstract this meeting).

1078495

IDENTIFICATION AND CHARACTERIZATION OF A NON-*E. COLI*-LIKE PROMOTER FROM *STREPTOMYCES*: Monica Ranes and Janet Westpheling, Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts

We have identified and characterized the promoter region of the endoglycosidase H gene of *Streptomyces plicatus*. *endoH*, whose product is a glycosidic enzyme secreted by *S. plicatus*, was cloned by Phillips Robbins and his collaborators (JBC 256: 10640). S1 nuclease protection analysis with RNA isolated from *S. plicatus* indicated that transcription of *endoH in vivo* originates at a site (indicated in the sequence below) located approximately 140 bases upstream from the beginning of the *endoH* coding sequence. To identify components of the transcriptional machinery which transcribe *endoH*, we used this gene as a template in *in vitro* transcription assays with partially purified *Streptomyces* RNA polymerase. It has recently been determined that RNA polymerase is heterogeneous in *Streptomyces* (Westpheling, poster this meeting) and at least two holoenzyme forms have been purified using as templates for transcription the *veg* and *ctc* promoters from *Bacillus subtilis*. We found that a form of polymerase which copurifies with the *ctc* transcribing activity transcribes the *endoH* gene *in vitro* and that transcription *in vitro* was initiated at or near the start site of RNA synthesis utilized *in vivo*. The nucleotide sequence of this region (determined by the method of Maxam and Gilbert) reveals homology with the "-35" and "-10" sequences of the *ctc* promoter.

```
TTTCGAGGTTTAAATCCTTATCGTTATGGGTATTGTTTGTAATAGG      ctc
ATTGACTGATTGACGCGC  TTCCGGCGGGCAGGGGAGGCACGGTG      endoH
```

Bases in the *ctc* promoter which have been shown to be important for utilization by *B. subtilis* $E\sigma^{37}$ as judged by homologies to other $E\sigma^{37}$ promoters (oversized letters), methylation protection experiments (asterisks) (Moran *et al.*, JMB 162: 709), and nucleotide substitution experiments (triangles) (Talti and Moran, JMB 175: 285) are compared to bases of the *endoH* promoter at similar positions from the transcription start site (underlined).

1078496

ASM CONFERENCE ON GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL MICROORGANISMS

ABSTRACTS OF INVITED LECTURERS

30 SEPTEMBER - 3 OCTOBER, 1984

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1078497

1

RECOMBINANT DNA AND THE PRODUCTION OF SMALL MOLECULES. J. Davies, Biogen, Geneva, Switzerland

Genetic engineering techniques have been very successful in converting *E. coli* into an industrial microorganism for the production of a variety of pharmacologically active proteins. The same techniques, although valuable in dissecting biosynthetic pathways and their control, have had much less impact on the production and modification of naturally occurring low molecular weight compounds. I will analyse the traditional approaches to yield improvement of antibiotics, alkaloids, etc., and discuss ways in which recombinant DNA techniques might contribute to the development of commercially important secondary metabolites.

1078498

THE GENETIC MANIPULATION OF BREWERS' YEAST STRAINS

Graham G. Stewart
Labatt Brewing Company Limited
150 Simcoe Street
London, Ontario, Canada N6A 4M3

The requirements of a brewing yeast strain can be defined as follows: *"In order to achieve a beer of high quality, the yeast culture must be effective in removing the desired nutrients from the growth/fermentation medium, (i.e. the wort), it must impart the required flavour to the beer and finally, the microorganisms themselves must be effectively removed from the fermented wort after they have fulfilled their metabolic role"*. In terms of process and product optimization in the brewing process, the utilization of wort sugars and carbohydrates is of prime importance. With the advent of light (lite) beers, the subject of the regulation of carbohydrate metabolism by brewing yeasts has gained even greater significance. Wort contains the sugars sucrose, fructose, glucose, maltose and maltotriose, together with dextrin material. In the normal situation brewing yeast strains are capable of utilizing sucrose, glucose, fructose, maltose and maltotriose in this approximate sequence, although some degree of overlap does occur, leaving the maltotetraose (G4) and larger dextrin molecules unfermented.

Although an abundance of information continues to accumulate concerning the genetics and biochemistry of laboratory ("academic") strains of *Saccharomyces cerevisiae*, there is still a marked lack of data on the genetic constitution of industrial yeast strains. As polyploids or aneuploids that do not possess a mating type, these strains exhibit a low degree of sporulation and yield spores of low viability, properties that render genetic analyses extremely difficult. Nevertheless, by virtue of their polyploid nature, the industrial strains are extremely stable and as such are resistant to ploidy breakdown, cross-breeding and mutational forces.

To successfully manipulate brewing strains the techniques of mutation, hybridization, spheroplast fusion and liposome-mediated transformation have been employed. Treatment of brewing strains with benomyl, a commercial fungicide reported to induce mitotic chromosome loss, permitted recovery of recessive auxotrophic markers indigenous to these strains. Hybridization has been used primarily to construct strains suitable for use as partners in spheroplast fusions with industrial strains. The derived strains should meet the following criteria: 1) rapid fermenters, 2) capable of fermenting wort dextrin material (*DEX*), 3) lack undesirable fermentation off-flavours (*pof*), 4) ethanol tolerant, 5) osmotolerant, 6) capable of utilizing maltose in the presence of glucose (derepressed) and, 7) possess killer activity.

In order to obtain novel hybrids harbouring such desirable properties, crosses were performed between the aforementioned and industrial strains by employing two methods - rare matings that involve use of karyogamy defective (*kar*) strains and/or spheroplast fusion. In addition, yeast transformation experiments using yeast plasmid DNA and total native DNA encapsulated in liposomes were carried out. In regard to liposome-mediated transformation, liposomes or synthetic microcapsules prepared by reverse phase evaporation not only protected entrapped DNA from nuclease degradation, but also interacted strongly with yeast spheroplasts under conditions favouring somatic fusion and transformation.

1078499

4

Cloning and Expression of the Aspergillus awamori Glucoamylase Gene in Yeast.

J.H. Meade, P.C. McCabe, G.E. Cole, V.P. Wittman, R. Tal, D.H. Gelfand, J.P. Holland[†], M.J. Holland[†] and M.A. Innis. Molecular and Biological Research Division, Cetus Corporation, 1400 Fifty-Third St., Emeryville, CA 94608, U.S.A.
[†]Department of Biological Chemistry, Medical School, University of California, Davis, CA 95616 U.S.A.

The extracellular glucoamylase from Aspergillus awamori hydrolyzes starch at the α-1-4 and α-1-6 linkages to yield glucose and is thus an enzyme of choice in commercial saccharification processes. The structure and sequence of the Aspergillus awamori glucoamylase gene will be presented. Features of the 5'-promoter region and the structure of the four small introns located within the glucoamylase gene appear homologous to several characterized yeast genes. However, no expression of glucoamylase protein or mRNA was observed when Yep vectors carrying the glucoamylase structural gene and considerable 5'- and 3'-flanking sequence were introduced into strains of yeast. We have determined that the lack of expression results both from the inability of yeast to recognize the Aspergillus promoter and from failure of yeast to properly excise the Aspergillus introns. In order to express the Aspergillus awamori glucoamylase in yeast, we used oligonucleotide directed mutagenesis techniques and partial cDNA clones to delete the four introns and introduce a yeast promoter, ENO1, at the 5'- end of the gene. We have achieved expression of the glucoamylase gene in yeast. The glucoamylase that is produced is glycosylated and secreted to the extracellular medium. We will discuss the NH_2-terminal processing and glycosylation of this heterologous enzyme by yeast.

1078500

THE MLS RESISTANCE PHENOTYPE IN *Streptomyces*: METHYLASE SEQUENCE AND ENHANCED ANTIBIOTIC PRODUCTION IN CONSTITUTIVELY RESISTANT MUTANTS

Hiroo Uchiyama, Mark Mayford, and Bernard Weisblum
University of Wisconsin Medical School, Madison, WI 53706

Two questions are addressed in the work that will be reported: (1) Is it possible to demonstrate structural similarity between MLS resistance methylases of *Staphylococcus aureus* and *Streptomyces* despite extremes of GC content in their respective chromosomal DNAs? and (2) In view of the inducibility of MLS resistance in antibiotic-producing strains of *Streptomyces* that synthesize MLS antibiotics, will efficiency of antibiotic production be enhanced in constitutively resistant cells?

(1) As a model system for sequence studies we used the MLS resistance methylase structural gene from *Streptomyces erythreus* subcloned in plasmid pIJ43 by C.J. Thompson et al., Gene 20:51-62. (1982). The DNA sequence corresponding to the methylase structural gene was determined by standard methods. One open reading frame deduced from the DNA sequence was readily aligned with, and showed clear-cut similarities to the *S. aureus* plasmid pE194 reference methylase, as well as to two other reference methylases whose sequences have thus far been determined, namely, the methylases encoded by *S. sanguis* plasmid pAM77 and by a subcloned *B. licheniformis* strain 749 chromosomal DNA determinant. Sequence similarities were found distributed in parallel along the entire length of the methylases used for reference and, significantly, many occur as conserved clusters of 2 to 5 consecutive identical residues. The extent of similarity found in the MLS resistance methylases suggests that they all evolved from a common progenitor. Oligonucleotide probes corresponding to several conserved clusters have been synthesized for use as probes to identify methylase-containing clones in gene libraries prepared from other *Streptomyces*.

(2) As a model system for antibiotic production studies we used *Streptomyces lincolnensis* NRRL 2936 a strain that synthesizes lincomycin. We have shown that adenine methylation in 23S rRNA of *S. lincolnensis* is inducible by lincomycin added exogenously to the culture medium. Since *S. lincolnensis* synthesizes lincomycin toward the end of its growth cycle, we speculated whether cells entering the stationary phase of growth might first synthesize a low, subinhibitory concentration of lincomycin and that this low concentration synthesized endogenously might function as an autoinducer of lincomycin resistance to enable the cell to synthesize higher levels of antibiotic subsequently. We further reasoned that constitutively resistant derivatives, having bypassed the autoinduction step, would initiate lincomycin production with a reduced lag and possibly synthesize the antibiotic to a higher level. We therefore measured lincomycin production over a period of 7 days in parallel in a mutant of *S. lincolnensis* selected with maridamycin (a macrolide) and in the wild-type progenitor strain. It was found (a) that lincomycin production was detectable one day earlier in the mutant than in the wild-type (day 4 in the mutant versus day 5 in the wild-type), and (b) that lincomycin production in the mutant reached a maximum level 5-fold higher than the wild type.

1078501

Regulation of the Yeast CYC1 Gene

Leonard Guarente
Department of Biology
M.I.T.
Cambridge, Massachusetts

The CYC1 gene of Saccharomyces cerevisiae encodes the iso-1-cytochrome c, which is a part of the respiratory chain in the inner mitochondrial membrane. Regulation of CYC1 has been studied using lacZ gene fusions and in vitro mutagenesis to identify promoter and regulatory sequences acting in cis, and standard yeast genetics to identify loci encoding regulatory proteins acting in trans. These experiments have shown that two regions are required in cis for CYC1 expression--one is the TATA box sequence at -30 from the transcription start, and the other is an upstream activation site (UAS) at about -250. The latter region consists of two independently functioning sites, UAS1 at -275, and UAS2 at -225. Each site, when placed upstream of the TATA box of a heterologous yeast gene, confers activation subject to the physiological signals which givern control of CYC1 (heme and catabolite repression). Current experiments are directed at constructing base substitution mutations in the UASs, identifying proteins which bind to the UASs to mediate activation, and ultimately, solving the mechanism of upstream activation. This solution may also apply to enhancer and regulatory sites of mammalian genes.

1078502

THE ROLE OF PROTEASE LA (THE LON GENE PRODUCT) AND ATP IN PROTEIN DEGRADATION IN E. COLI

Alfred L. Goldberg, Lloyd Waxman, A. Satish Menon, Stephen Goff
Harvard Medical School, Boston, MA 02115

E. coli, like mammalian cells or certain organelles, rapidly degrade highly abnormal proteins as may arise by mutations, gene fusions, or biosynthetic errors. Many proteins cloned in bacteria are rapidly hydrolyzed, and this process can sharply reduce the yield of the cloned polypeptide. In E. coli, as in mammalian cells, such abnormal proteins are degraded by a soluble process that requires metabolic energy. Much has been learned about the enzymes that may contribute to this process. E. coli contain 6 soluble proteases capable of degrading globin or casein. These activities have been purified and named proteases Do, Re, Mi, Fa, So, and La. In addition, E. coli contain two membrane-associated endoproteases that hydrolyze casein, as well as two soluble endoproteases named Ci and Pi that degrade smaller polypeptides (e.g. insulin) and many exopeptidases.

The energy requirement for proteolysis in E. coli results from the ATP requirement for the activity of protease La. This novel enzyme is the product of the lon gene. It seems to catalyze the initial cleavages on protein substrates, while the other (ATP-independent) proteases are involved in subsequent steps in this pathway. lon mutants contain a defective protease La and show a decreased capacity for degrading abnormal proteins, while strains containing increased amounts of protease La hydrolyze such proteins especially rapidly.

Protease La has a number of unusual properties: 1) It contains an inherent ATPase and cleaves proteins and ATP in a coupled process. 2) Protein substrates stimulate ATP hydrolysis 2-4 fold. 3) Approximately two ATPs are utilized per peptide bond cleaved. 4) It is very large for a protease and contains four identical subunits (Mr=94,000). We have isolated a similar enzyme from liver mitochondria, which catalyzes the rapid breakdown of abnormal mitochondrial polypeptides.

Recently we have identified fluorogenic tetrapeptide substrates which are cleaved by protease La in an ATP-dependent fashion. The hydrolysis of these peptides (unlike the breakdown of proteins) requires only binding of ATP and not its hydrolysis. Use of the peptide substrates has allowed us to dissect partial enzyme reactions and to show that ATP-dependent proteolysis involves a multistep reaction cycle: ATP binding occurs initially and allows proteolytic function. Subsequently ATP hydrolysis occurs and leads to a temporary inactivation of the protease (until a new ATP is bound). This process must occur repetitively until small peptides are generated. One additional novel property of this enzyme is that binding of protein substrates induces enzyme activation (as shown by more rapid hydrolysis of fluorogenic peptides). This activation of the enzyme by potential substrates appears important in the regulation and selectivity of intracellular proteolysis.

Recently, we and others have shown that protease La is a heat-shock protein. Its transcription and the overall rate of proteolysis in E. coli are controlled by the htpR locus. Protease La content rises under various conditions that cause the production of large amounts of abnormal proteins within cells (e.g. exposure to high temperatures, amino acid analogs, or certain plasmids). Induction of this protease thus may help protect against accumulation of such abnormal polypeptides.

1078503

The Search for the Perfect *E. coli* Expression System or is there a Holy Grail

Allan R. Shatzman
Dept. of Molecular Genetics
Smith Kline & French Labs
Philadelphia, PA 19101

Over the past several years, the ability to manipulate and clone genes into plasmids for expression in heterologous systems has become almost common place. However, the ability to predict the level at which a gene will express or to consistantly increase low level expression of some of these genes has eluded us. In order to maximize gene expression, we are developing a series of vectors to express efficiently any gene coding sequence, eukaryotic, prokaryotic, or synthetic in origin in *E. coli*. We have studied the expression of a large number of gene products including alpha-1-antitrypsin, metallothioneins, influenza NS1, *sis*, *myb*, *myc* and *abl*, not only to permit the study of their function, but with the intent of using sequences from well expressed gene products to increase the expression of those which are more poorly expressed. Increased expression is also being studied *via* the use of vectors which provide tandem promoters, downstream terminators, and varied ribosome binding sites and through new defective lysogens and host cell mutants. The effects of translational coupling on gene expression, especially with regard to possible secondary structure in RNA, will be discussed as will the effects of codon utilization and position with regard to translational regulation.

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1078506

Isolation and analysis of Streptomyces promoters

G. R. JANSSEN and M. J. BIBB
John Innes Institute, Norwich

Selected DNA fragments that promote transcription in Escherichia coli have previously been shown to function also as promoters in S. lividans; promoter-active fragments isolated in S. lividans, however, have been observed to function poorly, if at all, in E. coli. Isolation and analysis of Streptomyces promoter regions will facilitate the identification of structural features necessary for transcription of Streptomyces genes. Plasmid vectors have been constructed in S. lividans for the selection and isolation of DNA fragments that promote transcription of either a promoter-less chloramphenicol acetyltransferase or kanamycin phosphotransferase gene. Transcriptional read-through into the phosphotransferase gene by vector promoters is minimized by the upstream placement of a transcriptional terminator from phage fd. Conveniently located restriction sites allow for direct sequence determination of promoter-active fragments.

The promoter-probe vectors have been used to localize transcriptionally-active regions of genes cloned from various species of Streptomyces. Promoter-probing of DNA fragments encoding resistance to neomycin (from S. fradiae), thiostrepton (from S. azureus), and viomycin (from S. vinaceus) have identified promoter-active regions that precede open reading frames with a codon usage and nucleotide distribution predicted for coding sequences of high G + C content; this, in itself, permits the reliable and simple recognition of coding sequences. High resolution S1-nuclease mapping has been used to identify precisely the transcriptional start sites within these regions. DNA sequences upstream of these sites do not show extensive homology to the consensus promoter sequences of E. coli or Bacillus.

1078507

Chloramphenicol-Inducible Gene Expression. S. Mongkolsuk, E.J. Duvall, N.P. Ambulos Jr., and P.S. Lovett. University of Maryland Baltimore County, Catonsville.

pPL703 (∿5 kb) is a promoter-cloning plasmid for Bacillus subtilis. The plasmid consists of a promoter-less gene, cat-86, inserted in pUB110 at a site that prevents vector initiated transcription. A 21 bp multicloning site linker located 144 bp upstream from cat-86 in pPL703 permits the cloning of promoters into unique EcoRI, BamHI, SalI and PstI sites. In promoter containing derivatives of pPL703 cat-86 expression is inducible by chloramphenicol. Inducibility is independent of the promoter which is chosen to activate the gene, and inducibility is retained when cat-86 is replaced with the lacZ gene. Sequences essential to inducibility therefore reside in a 144 bp segment located between the site of promoter insertion and the cat gene. Evidence has been obtained suggesting that chloramphenicol inducibility is due primarily to post-transcriptional regulation. A model is presented in which chloramphenicol-modified ribosomes interact with cat mRNA to block formation of secondary structure that would, in the absence of the drug, sequester the cat ribosome binding site. This model applies equally in explaining the inducibility by chloramphenicol of the cat gene in plasmid pC194.

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REPLICATION AND CHROMATIN ORGANIZATION OF YEAST CHROMOSOMAL DNA PLASMID VECTORS

John F. Scott, Department of Microbiology, Biotechnology Center and College of Medicine, University of Illinois, Urbana, Illinois 61801

A large proportion of plasmid vectors used as vehicles for recombinant DNA in yeast contain Autonomously Replicating Sequences (*ARS*'s) derived from yeast chromosomal DNA. As a model system for our investigation of the mechanism of replication of nuclear DNA in the yeast *Saccharomyces cerevisiae* we have chosen one such element *ARS1*. This replicator was first isolated by Stinchcomb and Davis as part of a 1453 base pair EcoRI restriction fragment of yeast chromosomal DNA which also contained a functional *TRP1* gene. We later demonstrated that when the *ARS1-TRP1* fragment was circularized at the EcoRI ends in the absence of other DNA, and returned to yeast selecting Trp+, a 100-200 copy per cell relatively stable plasmid was established. We found that the plasmid (TRP1 R1 Circle, YARp1, Yeast Acentric Ring plasmid 1) was organized in a nucleosome-like structure and was replicated under cell cycle control comparable to the bulk of yeast nuclear DNA. Subsequently Fangman and co-workers have shown that the plasmid replicated once per molecule per cell division in early S-phase at a time similar to that of the original chromosomal segment.

More recently we have investigated the micrococcal nuclease protected regions on the chromatin form of the plasmid and have found one such region of greater than nucleosome size at or near the *ARS1* site as determined by *in vitro* mutagenesis experiments done by our laboratory and others. We have purified milligram quantities of the chromatin form of the plasmid and are using it in several ways including as an antigen to raise and screen a panel of monoclonal antibodies against associated determinants. We have also constructed several new plasmids in *Escherichia coli* which make introduction of additional DNA segments into YARp1 facile, and have shown that the product of an added *URA3* gene is overproduced in an amount comparable to plasmid copy number and similar to the amount observed for *TRP1* product from YARp1. Further investigations of the stabilities, copy numbers and replicative properties of YARp1 and its derivatives have also been conducted.

1078510

PRODUCTION OF RECOMBINANT VACCINES FROM MICROOGANISMS. Dennis G. Kleid, Genentech Inc., 460 Point San Bruno Blvd., So. San Francisco CA 94080.

In the last three years genetically engineered microorganisms such as E. coli and S. cerivisiae (yeast) have been demonstrated to be of use as a source of antigens for vaccine production. The first demonstration of this was when an antigenic protein coded by the Foot-and-Mouth Disease Virus was expressed in E. coli, isolated from the organisms and shown to protect cattle and swine from viral challenge. Recently the Hepatitis B surface antigen was produced in yeast and shown to protect chimpanzees from Hepatitis and shown to induce antibody in humans. Although the examples cited are very limited in number, and as yet no large scale human or animal trials have been reported, it appears that a new era in vaccine production as begun. However, there are several problems that remain to be overcome. The key to a successful vaccine is efficacy not the inexpensive production of an antigen. Considerable attention must be placed on the quality, safety, purity, and potency of the vaccine product produced. One of the major difficulties with production of vaccines via genetically engineered microorganisms has been the recreation of viral antigenic sites from the largely denatured proteins produced. The three dimensional structure of these sites and the necessity of refolding the protein in such as way as to recreate these sites is a major obstacle. This report will review these problems and discuss how they were overcome with the Foot-and-Mouth Disease VP1, the Hepatitis B surface antigen and the Herpes type I and II glycoprotein D.

1078511

Strategies for cloning genes for antibiotic biosynthesis in Streptomyces

D. A. HOPWOOD
John Innes Institute, Norwich, U.K.

Three general strategies have been used for the cloning of antibiotic biosynthetic genes: (1) detection of an individual biosynthetic enzyme cloned into a convenient recipient strain such as S. lividans (examples: PABA-synthase[1] and phenoxazinone synthase[2]); (2) complementation of blocked mutants of an antibiotic-producing strain (undecylprodigiosin[3] and actinorhodin[4]); (3) mutational cloning in an antibiotic-producing strain (methylenomycin[5]).

The example of strategy (2) provided by the genes for the polyketide antibiotic actinorhodin in S. coelicolor A3(2) will be described in detail. Mutations blocked at various points in the biosynthetic pathway had already been mapped by plasmid-mediated conjugation to a cluster of chromosomal loci. A cloned DNA segment capable of complementing all classes of act mutants caused actinorhodin biosynthesis in a host, S. parvulus, not known to synthesise any polyketide antibiotic[4]. Recent work by F. Malpartida has localised the different classes of act mutations in relation to the restriction map of the cloned DNA by complementation tests with subclones, and has defined the approximate limits of the transcription units by mutational cloning using att-deleted ϕC31 vectors.

We have transferred various parts of the actinorhodin DNA to two strains that produce other isochromanequinone antibiotics and, in collaboration with groups at Ohio State University, Columbus (H. G. Floss and colleagues) and at the Kitasato Institute, Tokyo (S. Ōmura and colleagues), are seeking novel "hybrid" antibiotics produced by the clones.

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1078512

Development of Systems for Heterologous Gene Expression in Streptomyces.

JEFFREY T. FAYERMAN, MICHAEL D. JONES, and MARK A. RICHARDSON, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

Streptomyces are commercially important gram positive prokaryotic organisms with a complex life cycle. Despite their utility in the production of antibiotics relatively little is known about the genetics and molecular biology of these microorganisms. In order to successfully carry out heterologous gene cloning, it is necessary to develop systems for gene transfer and expression in Streptomyces species of industrial interest. In the area of gene transfer, our group has developed three different cryptic Streptomyces plasmids, pFJ103, pNM100, and pFJ275, into gene cloning vehicles. The ability of these plasmids to transform Streptomyces species of industrial interest has been studied and, in general, the host range is broad. In the area of gene expression, our group has used the Bacillus subtilis veg promoter to drive expression in Streptomyces. Both the Escherichia coli β-galactosidase and hygromycin phosphotransferase genes are expressed in S. ambofaciens when the veg promoter and ribosome binding site are located immediately upstream from the structural gene. Details concerning the desired elements for such expression have been studied using both natural and synthetic DNA sequences. With successful heterologous gene cloning it might be possible to develop new antibiotics and to modify the chemical structure of existing antibiotics.

1078513

Streptomyces phage vectors and the analysis of genes for primary and secondary metabolism and differentiation

K. F. CHATER

John Innes Institute, Norwich, U.K.

Temperate phages offer a range of special properties valuable in gene cloning and analysis. In the lytic mode, plaques develop rapidly and are well suited for screening either with radio active probes or using color tests (e.g. with lacZ-fusion experiments). Moreover, selections for insertion or deletion of DNA can be applied. All of these aspects have been exploited in the development of a series of vectors based on the wide host-range Streptomyces temperate phage øC31. However, most of the current use of the vectors exploits their alternative lysogenic life-style, when they can integrate at single copy level into a recipient's chromosome in various ways, thereby providing possibilities for genetic analysis which nicely complement those offered by plasmid systems.

The phage vectors generally contain selectable resistance genes and a variety of unique cloning sites. They have been useful in shotgun cloning and in analysis of cloned DNA. Their integration at the preferred chromosomal attachment site is useful in tests of dominance and complementation of cloned DNA. Alternatively, they may integrate at the region homologous with the cloned fragment, allowing: (1) disruption of transcription units; (2) activation of promoterless genes, such as lacZ or the viomycin-resistance gene vph, incorporated into the vector; (3) homogenotization, so that a chromosomally located allele is transferred to the vector; (4) the cloning of flanking sequences without using radioactive probes; and (5) the genetic mapping of cryptic genetic elements (for example, insertion sequences). The phage system was used by H. Ikeda to clone a gene (glk for glucose kinase) required for glucose repression; and along with many other techniques, to analyse cloned DNA of the glucose-repressible gyl (glycerol utilization) operon (Seno et al., Mol. Gen. Genet. 193, 119, 1984). By acquiring an understanding of glucose repression of primary metabolism we hope to elucidate its nature in secondary metabolism and differentiation. Genes for these functions have also been cloned and studied with the phage system. Thus 17 kb of cloned DNA involved in methylenomycin biosynthesis appears to contain at least three transcription units and to include a regulatory gene and a gene for methylenomycin resistance; and two regions (bldA and bldB) involved in aerial mycelium formation have been cloned and used in genetic analysis of bld mutants by J. M. Piret.

1078514

AMPLIFIED DNA: STRUCTURE AND SIGNIFICANCE - C. L. Hershberger and S. E. Fishman, Eli Lilly and Company, 307 East McCarty Street, Indianapolis, Indiana 46285

High frequency occurrences of amplified DNA have been observed in streptomycetes. Most examples of amplified DNA were observed initially in experiments involving preparation and regeneration of protoplasts or growth with intercalating dyes; however, cause and effect relationships have not been demonstrated. Relatively short DNA segments of 5-20 kb amplify to 300-500 copies per chromosome. The parental strain normally contains one copy or at most a few copies of the DNA segment that is reiterated in the Amplified DNA Sequence (ADS). The ADS contains tandem head to tail reiterations of the short segment; however, all copies of the reiterated sequence may be in a single block of amplified DNA or copies of the reiterated DNA my be distributed in several different blocks of amplified DNA. There are examples of chromosomal deletions associated with the occurrence of ADS. The tandemly repeated segment of DNA in ADS-Sfl from *S. fradiae* is deleted in an independent mutant that was isolated from the progenitor of the mutant containing ADS.

Stability is different for the various examples of ADS in streptomycetes. Some ADS's are unstable and exhibit gradually decreasing copy number during growth; however, ADS-Sfl is very stable. The apparent copy number seems to remain constant after prolonged growth, repeated subculturings, and reisolation of single colonies. P. Matsushima and R. H. Baltz (personal communication) isolated mutants from regenerated protoplasts of *S. fradiae* that had deamplified ADS-Sfl. We found that the mutants retained residual copies of tandem reiterations but they contained deletions extending into neighboring segments of the genome.

The Amplifiable Unit of DNA (AUD) is the single copy of unamplified DNA sequence corresponding to the reiterated segment in ADS. AUD-Sfl from *S. fradiae* is a 12.7 kb sequence containing a 2.2 kb directly Repeated Sequence (RS) at each end. The reiterated unit of ADS-Sfl is a 10.5 kb sequence containing a single copy of RS in each reiterated DNA segment. The structural relationships between AUD and ADS suggest that amplification could occur by unequal crossing over, rolling circle replication or disproportionate replication and recombinational resolution.

S. fradiae contains two additional copies or a total of four copies of DNA with homology to RS. The two additional copies of RS are within 15 kb of each other but they are separated from each other by a sequence that is not homologous to the internal sequence of AUD-Sfl. Analyses of the deletion mutants described above provide a preliminary map delineating the relative positions of all four copies of RS. The arrangement of the four RS's suggests the potential for chromosomal rearrangements such as amplifications, deletions, inversions and translocations.

1078515

Physical and Genetic Characterization of Actinophage SF1, A P1-Like Phage Isolated from *Streptomyces fradiae*

Shiau-Ta Chung and Stuart A. Thompson
The Upjohn Company
Kalamazoo, MI

The actinophage SF1, like P1 bacteriophage in *E. coli*, exists in its host as a plasmid prophage (pUC13) and transduces chromosomal and plasmid markers from one strain to another via generalized transduction. Frequencies of SF1-mediated transduction range from $2x10^{-4}$ to $3x10^{-6}$ per P.F.U. for auxotrophic markers and pIJ702, and 1 to 3 per P.F.U. for mini-plasmids derived from pUC13. The encapsulation of SF1 DNA begins at a *pac* site and proceeds sequentially by headfuls of DNA in one direction along a concatemer. Although the mechanism of formation of transducing phage particles for chromosomal markers may mimic *pac* gene encapsulation, a recombination-mediated mechanism appears likely for the high frequency transduction of pUC13 mini-plasmids. We have also determined by restriction and deletion analyses the relative locations of the *pac* site, the immunity region, the origin of prophage replication, a locus for clear plaque formation and non-essential regions. Finally, although the host range of SF1 is limited to neomycin-producing *S. fradiae*, we have been able to introduce pUC13-derived mini-plasmids into other *Streptomyces* sp.

1078516

GENETIC INSTABILITY: AMPLIFICATION, DELETION AND REARRANGEMENT WITHIN THE DNA

Hildgund Schrempf, Dalhousie University,

Dep. of Biology, Halifax,N.S. B3H 4J1 Canada

Many plasmidless and plasmid-containing strains of Streptomyces are genetically unstable. Variants having alterations in their secondary metabolism arise frequently and are likely to differ from the corresponding wild-type strains by characteristic changes within the chromosomal and if present, extrachromosomal DNA. The chromosomal changes which have been identified include deletions (i.e. the structural gene for tyrosinase, required for melanin formation), amplification and rearrangement of specific DNA sequences. Depending on the variant tested, the amplified sequences may represent 5-40% of the total DNA. So far, it is unknown whether these sequences code for functions, but it has been shown that they are neither ribosomal, t-RNA nor plasmid genes. These sequences (or parts of them) exist in several copies within the genomes of most strains of Streptomyces and within some other Actinomycetes. Different classes of reiterated sequences have been characterized and it will be of further interest to gain an understanding of their functions and their possible role in the variability of the Streptomyces genome.

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1078517

Cloning, expression and regulation of the phenoxazinone synthase gene from *Streptomyces antibioticus*

George H. Jones

Phenoxazinone synthase (PHS) is thought to be a key enzyme in the biosynthesis of actinomycin by *Streptomyces antibioticus*. The phenoxazinone synthase structural gene has been localized to a 2.4 kb fragment of *S. antibioticus* DNA. This fragment has been cloned using the plasmid vector, pIJ702, and high levels of PHS activity are detected after transformation of *S. lividans* with the resulting recombinant plasmid. This plasmid also functions as a template in a streptomycete coupled transcription-translation system and the 2.4 kb insert codes for the synthesis of a protein whose electrophoretic and immunological properties are indistinguishable from those of the 89,000 Mr subunit of PHS. The cloned PHS gene has been used as a probe to examine the regulation of PHS expression in *S. antibioticus*. Dot blotting experiments have shown that the normally observed increase in PHS activity during growth of *S. antibioticus* cultures results from the production of new mRNA for the enzyme, and that the repression of PHS activity in glucose grown cultures is also mediated at the transcriptional level. The size of the PHS message has been examined by Northern blotting techniques, and the direction of transcription of the PHS gene has been determined using end-labeled restriction fragments derived from the 2.4 kb structural gene.

The cloning experiments also resulted in the isolation of two other DNA fragments from *S. antibioticus* which are capable of inducing production of active PHS after transformation of *S. lividans* with the corresponding recombinant plasmids. These fragments do not overlap the 2.4 kb structural gene and do not code for PHS related proteins in the coupled transcription-translation system. Rather, they appear to activate a normally "silent" PHS gene in *S. lividans*. That gene has been identified by Southern blotting using the cloned *S. antibioticus* PHS gene as a probe.

1078518

A MECHANISM FOR THE SITE-SPECIFIC EXCISION AND INTEGRATION OF THE SLP1 ELEMENT OF STREPTOMYCES COELICOLOR: AND IMPLICATIONS FOR THE FORMATION OF AUTONOMOUS SLP1 PLASMIDS

Charles A. Omer and Stanley N. Cohen
Department of Genetics
Stanford University Medical School
Stanford, CA 94305

SLP1 is a genetic element in the Streptomyces coelicolor A3(2) chromosome which can be transferred to the closely related strain Streptomyces lividans, 66 where it exists as either a chromosomally integrated element or as an autonomous plasmid of from 10-14 kb(1). We have demonstrated that the integrated form of SLP1 ($SLP1^{int}$) is a 17 kb DNA element that appears to excise from the S. coelicolor chromosome and exist transiently as a plasmid during transfer to a S. lividans $SLP1^{-}$ strain(2). This transiently-existing plasmid can direct its site-specific integration into the S. lividans chromosome at a unique locus that corresponds to the original chromosomal location of $SLP1^{int}$ in S. coelicolor. Plasmids that contain the complete 17 kb SLP1 sequence were constructed, 1) by in vitro circularization of restriction endonuclease generated DNA fragments of chromosomal DNA carrying a tandem duplication of $SLP1^{int}$; 2) by molecularly cloning in the E. coli plasmid pACYC177 of a 17 kb BamHI restriction fragment that contains the complete $SLP1^{int}$ sequence. When these plasmids were used to transform S. lividans $SLP1^{-}$, the resulting transformants contained the SLP1 sequence chromosomally integrated at the same site as when the $SLP1^{int}$ sequence is transferred by mating. If additional DNA is inserted into the plasmid containing the complete SLP1 sequence, upon transformation the exogenously derived segment will integrate into the S. lividans chromosome along with the SLP1 DNA.

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The physically autonomous SLP1 plasmids obtained by mating S. coelicolor with S. lividans are structural subsets of the transiently existing 17 kb complete SLP1 plasmid. The largest SLP1 plasmid isolated by matings, SLP1.2, retains the 1.72 kb Pst I fragment that contains the attachment site for SLP1; but has deleted another region required for integration. The smaller SLP1 plasmids have lost both the attachment site and the region deleted in SLP1.2. We have constructed an SLP1 plasmid in which only the 1.72 kb Pst I fragment which contains the attachment site and an adjacent 0.4 kb Pst I fragment are deleted. This constructed plasmid also fails to integrate into the chromosome.

A model for the site-specific excision and integration of $SLP1^{int}$ and a possible mechanism for the formation of the autonomous SLP1 plasmids will be presented.

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1078520

Transport of Invertase from the Endoplasmic Reticulum to the Golgi Body in Yeast

Randy Schekman, Irene Schauer, and Anton Haselbeck

Department of Biochemistry
University of California, Berkeley

Secretion and cell surface growth have been analyzed in S. cerevisiae by the isolation and characterization of conditionally-lethal mutants that are specifically blocked in these processes. Mutations in 23 genes have been obtained which cause secretory organelles (endoplasmic reticulum, Golgi bodies, secretory vesicles) and glycoproteins to accumulate at a nonpermissive growth temperature. An additional two genes are required for translocation of newly-synthesized polypeptides across the ER membrane. Mutants blocked early in the pathway have shown that glycoprotein carbohydrate synthesis is compartmentalized: N-glycosidic core oligosaccharides are added in the ER, and outer chain oligosaccharides are added in the Golgi body. Part of the pathway defined by these mutants is also required for the assembly of the yeast vacuole. Secretory and vacuolar proteins are transported from the ER to the Golgi body where they are sorted.

The role of structural signals in intercompartmental transport has been addressed by the isolation of yeast invertase (SUC2) mutations that result in delayed secretion. Retention of full enzyme activity in a secretion-defective mutant invertase was used to distinguish transport lesions from more general perturbations of protein structure. Two mutations that delay transport of core glycosylated invertase, but not acid phosphatase, from the endoplasmic reticulum have been mapped in the 5' coding region of SUC2. One mutation (SUC2-s1) converts an ala codon to val at position -1 in the signal peptide; the other (SUC2-s2) changes a thr to an ile at position +64 in the mature protein. Mutation s1 results in slow processing of the signal peptide which can account for the ~50 fold reduced rate of transport. s2 Mutant invertase is transported 7 fold more slowly than normal, with no delay in signal peptide cleavage, and no detectable abnormal physical property of the enzyme.

A functional assay for intercompartmental transport of invertase in vitro has been developed. The assay is based on a specific carbohydrate modification, the addition of α-1,3 mannose units to the outer chain oligosaccharide, that occurs when invertase is transported to the Golgi body. An antibody directed against this linkage will precipitate active invertase, although the modification is not essential because fully active invertase is secreted by a mutant (mnn1) that is defective in the α-1,3 mannosyltransferase. Core-glycosylated invertase, accumulated in the ER in a pleiotropic sec mutant at 37°C (sec18), provides a source of ER donor membranes. In order to ensure an intercompartmental reaction, the donor cell also carries the mnn1 mutation, and a recipient cell that is sec18 (or SEC18) MNN1 and ΔSUC2 (invertase gene deleted) is used to provide a distinct Golgi membrane. Transport from the donor to the recipient must occur for invertase to be converted to a form that is precipitated by the α-1,3-man antibody. At 24°C about 2 to 4% of the ER-bounded invertase is converted in a reaction that requires GDP-mannose and ATP, while at 30°C or 37°C no transport is detected. sec18 Mutant cells are similarly temperature sensitive. Furthermore, the reaction is temperature sensitive when recipient membranes are derived from sec or SEC cells, suggesting that the sec18 lesion is in the donor membrane. Donor-recipient mutant membrane combinations of the other 9 sec genes defective in transport from the ER are being examined to see which can be complemented. Success in this will allow purification of functional SEC gene products.

1078521

Protein Secretion in Bacillus and *E. coli*: Similarities and Differences

J.O. Lampen, P.S.F. Mézes and M. Hussain
Waksman Institute of Microbiology
Rutgers - The State University of New Jersey
Piscataway, NJ 08854

Although *Bacilli* are efficient secretors of proteins, information on the mechanism of secretion is largely derived from comparisons with the process in Gram-negative bacteria. A major handicap is the paucity of mutants with a generalized defect in protein transport. The types of proteins secreted by *Bacilli* and their ultimate locations will be reviewed as well as the available evidence for a secretory apparatus. The signal sequences of *Bacilli* differ from typical sequences in Gram-negative organisms in two ways: 1) The N-terminal segment is more positively charged and frequently longer, and 2) the hydrophilic stretch of small and polar residues continues beyond the probable signal peptide with further processing occurring here to form the mature extracellular protein. For the few identified products of signal peptidase action the sequences involved are consistent with the requirements for cleavage in Gram-negatives. Both a general and a lipoprotein-specific enzyme are present. Secreted proteins are synthesized on membrane bound ribosomes, and transport and processing can be coupled to translation. Nevertheless the relative resistance of some emerging proteins to external proteases implies the existence of stable structure at this stage and emergence relatively late in the growth of the nascent chain. Processing by proteases is both an essential step in maturation and a major impediment in production of cloned foreign proteins. Factors affecting proteolysis will be discussed.

1078522

Membrane Assembly and Bacterial Protein Export

We have studied the process by which proteins of the E. coli cell surface are exported through the plasma membrane. Several shared elements in this export process have been discovered: an amino-terminal leader sequence, a requirement for the membrane electrical potential, receptor proteins, and an endo-proteolytic processing by membrane bound leader peptidase. In order to study these events at a chemical level, we have cloned the gene for leader peptidase, purified the leader peptidase itself, and isolated chemically and radiochemically pure M13 procoat protein. Assembly events have been reconstituted using these components in a liposomal reaction. These studies, and those from other laboratories, have shown that protein export in bacteria cells shares features ascribed to protein translocation in endoplasmic reticulum and mitochondria in eukaryotic cells.

1078523

EXPORT OF PROTEIN IN BACTERIA

Linda L. Randall[1], Eva Murén[1] and Evert P. Bakker[2]

[1]Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164-4660

[2]Fachgebiet Mikrobiologie, Universität Osnabrück, FRG

Translocation of protein across the cytoplasmic membrane during secretion into the periplasmic space is independent of elongation of the polypeptide in *E. coli*. Thus we cannot invoke the energy of protein synthesis as the driving force in passage of the protein through the membrane. Since secretion in bacteria requires proton motive force, the cell might utilize the electric component of proton motive force to drive transfer. Data will be presented indicating that the energy requirement is satisfied by the total proton motive force with no specificity for either the membrane potential or the pH gradient. Mechanisms that exclusively utilize the electrical potential are not consistent with these observations. Alternatives must be considered.

1078524

RECOMBINANT DNA STUDIES IN CEPHALOSPORIUM ACREMONIUM. S. W. Queener, T. D. Ingolia, P. L. Skatrud, J. L. Chapman, K. R. Kaster, Eli Lilly and Company, Indianapolis, Indiana 46285

The fungus, Cephalosporium acremonium, is used in commercial production of cephalosporin C (CPC). CPC is an intermediate in the manufacture of at least eight different clinically useful cephalosporin antibiotics. A transformation system was developed for C. acremonium which will allow directed construction of strains with characteristics that enhance the manufacture of clinically useful cephalosporin antibiotics.

Plasmid pIT221 was constructed from nine starting materials. Genetic elements in pIT221 include sequences from pBR322, which allow replication and selection in E. coli, an autonomous replication sequence (ARS) from C. acremonium, which functions in yeast, [Current Genetics (1984)8:155-163] and a hybrid antibiotic resistance marker [Current Genetics (1984)8:353-358]. The hybrid gene was constructed by splicing the promoter region of the phosphoglycerokinase (PGK) gene from Saccharomyces cerevisiae to the protein-coding region of a hygromycin-phosphotransferase (HPT) gene from plasmid pKC203 (in E. coli ATCC 31912).

Conditions were developed which allowed C. acremonium (ATCC 11550) protoplasts to take up pIT221 DNA, regenerate cell walls, and express the HPT protein-coding sequence. Important parameters for successful transformation include the conditions used to prepare cells for protoplasting, time allowed to digest cell walls, concentration of PEG in the transformation mixture, and temperature at which regeneration is carried out. Expression was easily detected by overlaying with hygromycin B to a final plate concentration of 100 mcg/ml which killed all cells not exposed to pIT221 DNA. When 2-3 x 10^8 protoplasts in a 10 ml transformation mixture were exposed to 20 mcg of pIT221 DNA, between 1 and 16 hygromycin-resistant transformants (total 92) were regenerated on selective medium and subcultured in 11 of 14 experiments. Abortive transformants which failed to transfer on subculture to fresh selective medium were also observed. Southern analyses were performed with three transformants and the untransformed wild type using [^{32}P]pBR322 and a second ^{32}P-probe that allowed detection of the bacterial HPT protein coding sequences. Each of the C. acremonium transformants contained pBR322 sequences and HPT sequences in their high molecular weight (HMW) DNA. DNA of the untransformed wild type did not contain any sequences that hybridized to either probe. In vitro HPT assays were performed on ATCC 11550 and transformants derived from ATCC 11550 (See Abstract #134, "Phosphotransferase Activity in C. acremonium, J. Chapman and S. W. Queener) and independently confirmed the transformation events. Restriction digestion of total DNA from transformant CPC-T1 and Southern hybridization of the resulting DNA fragments indicated multiple inserts of pIT221 DNA in the HMW DNA of the fungal transformant. CPC-T1 retained its ability to produce CPC in a fermentor containing medium without hygromycin B. All cells sampled at harvest were still resistant to hygromycin B.

Modifications of pIT221 designed to increase transformation frequency in C. acremonium have been conducted (See Abstract #133, "Construction of Plasmids Containing C. acremonium Ribosomal DNA", P.L. Skatrud et. al.).

1078525

DEVELOPMENTALLY REGULATED EXPRESSION OF THE ASPERGILLUS NIDULANS SPOC1 GENE CLUSTER. B.L. Miller, K.Y. Miller and W.E. Timberlake, University of Calif., Davis, Calif. The SpoC1 gene cluster is a 37 kb region of the Aspergillus nidulans genome which codes for at least 19 developmentally regulated poly(A)+RNAs and is bordered by 1.1 kb direct repeats. Transcripts from the central portion of the cluster (with one exception) appear late in conidiophore development and accumulate specifically in asexual spores. However, those transcripts encoded at the borders of the cluster are detected in both somatic cells and spores. These results indicate that a chromatin level control mechanism operating over the SpoC1 domain may be one aspect of the coordinated expression of genes within the cluster (Gwynne, B. Miller, K. Miller and Timberlake, J. molec. biol., in press). We have begun an investigation of the mechanisms regulating expression of the cluster by taking advantage of DNA-mediated transformation of A. nidulans to 1) relocate strongly regulated genes from the cluster to other chromosomal positions, 2) create disruptions in the cluster, 3) create mutations in genes within the cluster via gene replacement and 4) introduce a non-developmentally regulated gene (arg B) into the SpoC1 region via cluster disruption. Preliminary results indicate that chromosomal position may be involved in the correct regulation of at least one of the two genes from the cluster, although the differential accumulation of this transcript in spores versus hyphae indicates that a position-independent component of regulation is also involved. Disruption of the SpoC1 cluster by the insertion of 10.2 kb of DNA does not appear to affect the correct regulation of those genes located on either side of the insert.

1078526

The Role of mRNA Structure in the Regulation of Protein Synthesis: Implications for Industrial Genetic Engineering

John N. Vournakis and Calvin P. H. Vary
Department of Biology, Syracuse University, Syracuse, New York

A method is described for the experimental determination of the secondary structure of RNA using enzymatic and chemical cleavage data coupled with computer analysis. The structure-specific enzymes S1 nuclease, T2 RNase and cobra venom ribonuclease are used to locate nonpaired and base-paired nucleotides. New information obtained with the intercalating agent methidium-propyl EDTA-Fe (II), which binds to base-paired nucleotides and cleaves RNA in the presence of DTT, is analysed by a computer-based microdensitometric technique to provide great detail in identifying double stranded regions. Computer techniques that utilize the enzymatic susceptibility information to generate a minimum free-energy structure are used to obtain secondary structure models. A second method, using acrylamide-agarose gel electrophoresis, is described for the determination of the relative protein synthesis initiation rates of end-labeled eukaryotic mRNAs. These methods are applied to a number of small prokaryotic RNAs and to the rabbit globin mRNAs as an example of a general approach for relating RNA structure and function. A discussion of the role of messenger RNA structure in the regulation of translation is included with an emphasis on applications to production of proteins by genetic engineering.

1078527

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