DOE/ER/20072-1

PLANT RECOGNITION OF *BRADYRHIZOBIUM JAPONICUM* NOD FACTORS

Final Report

for period 9-15-92 to 3-14-97

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January, 1998

Prepared for

THE U.S. DEPARTMENT OF ENERGY
AWARD NO. DE-FG05-92ER20072

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1. Project Title: Plant recognition of *Bradyrhizobium japonicum* nod factors.

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3. DOE award no.: DOE DE-FG05-92ER20072

4. Period covered by this report: Sept. 15, 1992 to March 14, 1997

5. Unexpended funds remaining: none

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7. Final project report:

This grant had three objectives: 1. Isolate and identify the unique *nod* factor metabolites made by different wild-type *B. japonicum* strains; 2. Investigate the biological activity of these unique *nod* factors, especially as it relates to host range; and 3. Initiate studies to define the mechanism of plant recognition of the *nod* factors. As described below, we have completed objective 1, largely completed objective 2, and have made significant progress on objective 3.

Goal I. Isolate and identify the unique *nod* factor metabolites made by different wild-type *B. japonicum* strains. This objective has now been completed. The manuscripts summarizing these results are:

Carlson, R.W., J. Sanjuan, U.R. Bhat, J. Glushka, H. Spaink, A.H.M. Wijfjes, A.A.N. van Brussel, T. J.W. Stokkerman, K. Peters, and G. Stacey. 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. 268: 18372-18381.

Luka, S., J. Sanjuan, R. W. Carlson and G. Stacey. 1993. *nolMNO* genes of *Bradyrhizobium japonicum* are co-transcribed with *nodYABCSUIJ* and *nolO* is involved in the synthesis of the lipo-oligosaccharide nodulation signals. J. Biol. Chem. 268: 27053-27059.

Stacey, G., S. Luka, J. Sanjuan, Z. Banfalvi, A.J. Nieuwkoop, J.Y. Chun, L.S. Forsberg, and R. Carlson. 1994. *nodZ*, a unique host-specific nodulation gene, is involved in the fucosylation of the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. J. Bacteriol. 176: 620-633.

In addition to wanting to identify the *nod* factors from *B. japonicum*, we had additional motivation for pursuing the work under this objective. First, we wanted information about the *nod* factor structures as an aid to further studies to define the biochemical functions of the various *nod* gene products. For example, Stacey et al. (18) presented data that the *nodZ* gene product is involved in fucosylation of the *nod* factor. Secondly, as described in Stokkermann et al. (22), we noticed a correlation between the pattern of *nod* factor production and genotype specific nodulation. That is, *B. elkanii* (previously called *B. japonicum* group II) strain USDA61 is restricted for nodulation on soybean lines containing the Rj4 allele. However, mutants of this strain can be isolated that overcome this restriction. These mutants show a marked variation in their profile of *nod* factor metabolites. We hypothesized that specific changes in the *nod* factors produced could explain the change in host range. For example, it is possible that specific *nod* factors produced by the wildtype could be inhibitory to nodulation on Rj4 soybean. We sought to isolate the *nod* factors from the wildtype and mutants in order to test this hypothesis. As described in the experimental portion of this grant, we now have in our possession chemically synthesized and natural produced *nod* factors to explore the possible mechanism of Rj4 exclusion of nodulation. Thirdly, we wanted to elucidate the structure of the various *B. japonicum nod* factors to support our collaboration with Dr. Tomoya Ogawa with regard to chemically synthesizing *nod* factors to confirm their biological activity and to explore structure/function relationships (see below).

We have identified 13 different *nod* factor metabolites produced by *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* strain USDA61. In addition to these compounds, we also identified BjNodV (18:1) as the compound produced by a NodZ or NolO mutant and BjNodIV (16:0, Gro) and BjNodV (16:0) produced by a NolO mutant of *B. japonicum* strain USDA110. In addition to addressing the questions listed above, an added bonus of these studies was the discovery of metabolites substituted on the reducing sugar by glycerol. We suspect that these metabolites, isolated from *B. elkanii* strain USDA61 and mutants of *B. japonicum* strain USDA110, may represent intermediates in the synthesis of the *nod* metabolites (see below).

Mutants of *B. elkanii* strain USDA61 capable of efficient nodulation of Rj4 soybean lack the NodBeIV(Cb,$C_{18:1}$,MeFuc), NodBeIV(Cb,$C_{18:1}$,Fuc,Gro), NodBeIV(Cb,$C_{18:1}$,NMe,Fuc,Gro), NodBJIV($C_{18:1}$,NMe,Fuc,Gro), NodBeIV($C_{18:1}$,Fuc,Gro) and NodBe(Ac,Cb,$C_{18:1}$,MeFuc) *nod* factors. Therefore, these factors may be involved in inhibiting nodulation on Rj4 soybean roots.

Goal 2. Investigate the biological activity of these unique *nod* factors, especially as it relates to host range. Significant progress has been made relative to this objective, but work is continuing. Manuscripts relating to this portion of the work are:

Stacey, G., S. Luka, J. Sanjuan, Z. Banfalvi, A.J. Nieuwkoop, J.Y. Chun, L.S. Forsberg, and R.

Carlson. 1994. *nodZ*, a unique host-specific nodulation gene, is involved in the fucosylation of the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. J. Bacteriol. 176: 620-633.

Stokkermans, T.J.W., S. Ikeshita, J. Cohn, R.W. Carlson, G. Stacey, T. Ogawa, and N.K. Peters. 1995. Structural requirements of synthetic and natural product lipo-oligosaccharides to induce nodule primordia on *Glycine soja*. Plant Physiol. 108: 1587-1595.

At the time of our initial studies, it was surprising to find that *B. japonicum* produces such a wide variety of *nod* factor structures. Our hypothesis is that this variety arises from two factors; 1. some of the factors isolated are biosynthetic intermediates and 2. a diversity of *nod* factor metabolites is required for nodulation of a broad range of host plants. In order to address the latter possibility, it is necessary to test the various purified factors on a variety of host plants. Our original paper describing the structure of the BjNodV($C_{18:1}$,MeFuc) compound showed that this factor was capable of inducing root hair curling on soybean (14). A variety of the *nod* metabolites produced by *B. japonicum* and *B. elkanii* will induce root hair deformations on *Vicia hirsuta*, a particularly promiscuous and sensitive plant for such bioassays (2). We recently sent the purified NodBjV($C_{18:1}$,MeFuc) to Bill Broughton and his lab has published that it induces root hair curling on siratro (*Macroptilium atropurpureum*), an alternative host for *B. japonicum* (12). Moreover, this compound also induces a rapid and transient alkalinization of the culture medium when added to suspension-cultured tomato cells (19). This latter bioassay system is commonly used to test for the ability of compounds to elicit reactions involved in the plant defense response. Such active compounds are called elicitors. Therefore, these results suggest that the *B. japonicum nod* factor can be recognized as an elicitor on plants other than soybean.

Stacey et al. (18) reported that the NodBjV($C_{18:1}$,MeFuc) compound could induce cortical cell division when applied in nM amounts to soybean roots. The NodBjV($C_{18:1}$) compound produced by a NodZ mutant did not possess this activity. Thus, it would appear that fucosylation of the terminal, reducing sugar of the *nod* factor is required for this biological activity (see below for exceptions). However, the type of nodule structures reported in this paper did not closely resemble true nodules. As part of our continuing collaboration with the laboratory of Dr. Kent Peters, his laboratory carried out experiments to test the biological activity of the *nod* metabolites purified from *B. elkanii*. The nodule structures observed were far superior to what we were able to obtain in Knoxville (21). After a few phone calls, we were able to repeat these results and now routinely obtain very nice nodule structures by the addition of purified *B. japonicum nod* factors. The structures produced have lateral vascular tissue and appear quite similar to normal nodules.

As part of a continuing collaboration with Dr. Tomoya Ogawa, we have obtained ug quantities of several chemically synthesized *nod* factors. Or preliminary results are quite surprising. First, our data are the first to show that the synthetic molecules have the same activity as the purified *nod* factor. This is an essential confirmation that the purified molecules are biologically relevant. Second, there is a remarkable correlation between the specificity for hair deformation (Had) and the induction of cortical cell division (Noi). This, in spite of the fact, that the cortical cell division assays were carried out at Ohio State Univ. and the hair deformation assays were carried out at

the Univ. of Tennessee. The results of both assays were not shared until all of the assays were complete. Third, unlike data from studies with *nod* factors active on vetch and alfalfa (4,17), there is only a slight impact on biological activity of variation in the lipid moiety. Fatty acids of C18:1Δ11, C18:1Δ9, and C16:0 all produce biologically active molecules. Additional synthetic derivatives will have to be synthesized to further explore the importance of the fatty acyl residue to biological function. Fourth, there is a novel interdependence of backbone length and reducing-end modification that has not been previously reported. For alfalfa, there is a dependence on the length of the oligosaccharide with the tetramer of NodRmIV($C_{16:2}$,S) being 100-times more active than the pentamer, NodRMV($C_{16:2}$,S), in inducing nodule primordia (15). However, sulfation of the terminal, reducing sugar is absolutely required. As reported previously (18) and confirmed by our results, fucosylation of the NodBj($C_{18:1}$,MeFuc) compound is also required for biological function. However, the importance of fucosylation varies depending on the length of the oligosaccharide backbone. Atetramer substituted only with a $C_{16:0}$ fatty acid has significant biological activity in both the Had and Noi assays. These results are confirmed by analyzing one of the minor *nod* factor components purified from induced *R. leguminosarum* bv. viceae, NodRlIV($C_{18:1\Delta11}$) (provided by Herman Spaink). The data indicate that this compound also has appreciable activity when assayed on soybean.

One way to reconcile these data is to propose that an acylated, chitose tetramer is the minimum biologically active molecule. Additional additions or modifications to this structure may enhance activity or provide a protective function. For example, it has recently be suggested that sulfation of the *nod* factor produced by *R. meliloti* is important in protecting the molecule from degradation by plant produced chitinases (20). Fucosylation of the *B. japonicum nod* factors may play a similar role. The real enigma created by the data is what role do the various chemical substitutions play in host specificity? For example, if *R. leguminosarum* bv. *viciae* produces, even in minor amounts, a compound active on soybean, why can't this organism nodulate soybean? Clearly, we have more to learn about the role of *nod* factors and other possible factors that might control host range. One possibility mentioned above is that specific *nod* factors may inhibit nodulation on particular host cultivars or species. Therefore, it may not only be important that the organism produce an active *nod* factor, but also that it produce a profile of factors that favor (and not inhibit) nodulation of a particular host. Our possession of purified and synthetic *nod* factors will allow us to examine some of these questions.

The recent publication of Staehelin et al. (19) showing that rhizobial *nod* factors can be recognized as elicitors by tomato cells also suggests that a receptor for *nod* factor may be a general property of plants. Our data suggest that a relatively simple acylated oligochitose structure is sufficient for biological activity on soybean. Such a structure resembles known chitose elicitors produced by plant pathogenic fungi (1, 13). Taken together these data suggest that the *nod* factor specificity may not lie in receptor recognition but in the stability of the *nod* factor in the plant or in some step subsequent to *nod* factor reception. Therefore, on-going work on chitose receptors in plants may be directly applicable to an understanding of *nod* factor function (e.g., 16).

Goal 3. Initiate studies to define the mechanism of plant recognition of the *nod* factors. This work has been initiated and is continuing. We are focusing on two aspects:

1. An investigation of the biological activities of natural and synthetic analogs of the *B. japonicum* nodulation factors.
2. An investigation of the molecular plant response to nodulation factors using known nodulin probes and also novel nodulin genes recently isolate in our laboratory.

A. Investigation of the plant response to natural and synthetic *nod* factors utilizing known nodulin gene probes. We now have in the laboratory soybean cDNA clones to the following early nodulins (i.e., expressed within 5 days of bacterial inoculation) and other genes known to be expressed during nodulation: ENOD2, ENOD40 a and b, NOD#315, ENOD#93, NOD#70, ENOD55, Leghemoglobin a and c, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), NOD24, NOD26, cdc2 and cyclin B. These genes have been kindly provided to us from a number of laboratories (i.e., Bisseling, Kouchi, Sengupta-Gopalan, Gresshoff). We are now in the process of examining the expression of these genes in response to inoculation with various naturally purified and chemically synthesized *nod* factors. Due to the limited quantities of *nod* factor which results in low amounts of nodule tissue, we are using a semi-quantitative RT-PCR assay to measure gene expression (c.f., 6). Our initial studies indicate that ENOD2 is expressed within 48 hours after addition of NodBj($C_{18:1}$,MeFuc). ENOD2 expression can still be detected 13 days after addition of the *nod* factor. In addition, we also detect ENOD40 expression by 48 hours. However, these latter assays are complicated by the fact that ENOD40 mRNA can be detected in all plant tissues. This result is consistent with the published report by Kouchi and Hata (31), but contradicts the published report by Yang et al. (23). However, we are using the PCR primers described in Kouchi and Hata (7) and this may explain the discrepancy. As discussed below, we hope to continue this work and to also use the various probes to analyzed the plant response to the various *nod* factor structural variants that we possess.

Figure 1 shows very preliminary results with regard to in situ hybridizations of an ENOD40 anti-sense probe to nodule soybean nodule sections and nodule-like structures induced with the purified NodBj-V($C_{18:1}$,Mefuc) factor. In this case, the Nod factor-induced structure is not well developed and should not be taken as indicative of the type of response normally seen. These data are included solely to make two points; one, we are seeing ENOD40 expression and in a manner consistent with its known tissue specificity and two, our collaboration with Dr. Kouchi is developing well and yielding interesting results. Publication quality data will come when we provide Dr. Kouchi with more suitable plant material to study.

B. Isolation of novel early nodulin genes from soybean. Our reading of the literature with regard to early nodulin genes in pea, alfalfa, and soybean, the three primary plants studied, is that only a narrow repertoire of genes have been isolated. Moreover, only a few of the genes reported appear to be expressed very early (<24 hours) after inoculation. For example, the expression of ENOD2 can be detected by Northern analysis within 4 days after inoculation and within 3 days by Southern hybridization to cDNA amplified by RT-PCR (reverse transcriptase-polymerase chain

reaction) (7). ENOD12, which also encodes a proline-rich protein, can be detected by Northern blotting within 48 hours after inoculation of alfalfa plants. In transgenic alfalfa plants containing an ENOD12-*gusA* fusion, induction of β-glucuronidase activity was first detectable within 3 to 6 hours after inoculation (11). Clones of soybean genes showing a similar rapid response have not been isolated. It is our view that such genes could identify important steps in the primary plant recognition events leading to subsequent nodule organogenesis. Therefore, we have undertaken an effort to isolate plant genes induced very rapidly (<48 h) after inoculation.

The method we have chosen to isolate such early nodulins makes use of the 'differential display' method published by Pardee and colleagues (8-10). This method involves the isolation of mRNA from uninoculated soybean cv. Bragg plants and from inoculated soybean cv. NTS382 plants. The latter cultivar is a near-isogenic mutant line of cv. Bragg that has the supernodulating phenotype (3). Previous studies indicated that the molecular response of cv. NTS382 to bacterial inoculation was similar to the wildtype, but the amount of nodule-specific mRNA was considerably higher, consistent with the formation of more nodule tissue (5, P. Gresshoff, personal commun.). Therefore, we have chosen to compare the uninoculated Bragg and inoculated NTS382 in order to enhance the detection of induced genes. We are cognizant of the fact that we might also isolate genes involved in the supernodulating phenotype, but these would also be of interest. The two preparations of mRNA are amplified by RT-PCR using poly-dT and a random 10mer primer. The cDNA is then electrophoresed and the banding pattern of the two preparations directly compared. Bands that are different are reamplified using the same primer and then cloned. The resulting clones are used to confirm the regulatory pattern by Northern hybridization.

At present, we have utilized 35 unique, random 10mer primers and obtained 14 putative clones encoding genes up-regulated upon inoculation. This work is still in the early stages, but two clones are, at present, of particular interest. One clone, #825, encodes a cDNA fragment of 250bp and appears, based on Northern hybridizations, to be induced within 12 hours after inoculation but is no longer expressed by 48 hours. To our knowledge, this type of rapid and temporal expression upon inoculation has never been reported before. Clone #826 also exhibits a pattern of temporal expression but first appears 24h after inoculation and disappears by 72 h. We are still in the process of analyzing the additional clones. These genes will be useful in analyzing the plant molecular response to *nod* factor addition.

Host-specific nitrogen fixation. As part of the previous grant, we continued our investigation of additional bacterial genes that might play a role in host specificity. The attached manuscript

Chun, J.-Y., G. Sexton, E.Roth, and G. Stacey. 1994. Identification and characterization of a novel *Bradyrhizobium japonicum* gene involved in host-specific nitrogen fixation. J. Bacteriol. 176: 6717-6729.

describes an aspect of this work. Originally we thought that the region identified in this manuscript might be involved in nodulation specificity and/or *nod* factor synthesis. However, further analysis found that this region encoded a gene that was essential for nitrogen fixation on

cowpea, but not on other hosts. This is very interesting work, but not directly pertinent to the focus of the present proposal.

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9. Publications resulting from this work:

Carlson, R.W., J. Sanjuan, U.R. Bhat, J. Glushka, H. Spaink, A.H.M. Wijfjes, A.A.N. van Brussel, T. J.W. Stokkerman, K. Peters, and G. Stacey. 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. 268: 18372-18381.

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Chun, J.-Y., G. Sexton, E.Roth, and G. Stacey. 1994. Identification and characterization of a novel *Bradyrhizobium japonicum* gene involved in host-specific nitrogen fixation. J. Bacteriol. 176: 6717-6729.