Abnormal Bacteroid Development in Nodules Induced by a Glucosamine Synthase Mutant of *Rhizobium leguminosarum*

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Mutation of the chromosomal gene (*glmS*) encoding glucosamine synthase in *Rhizobium leguminosarum* biovar *viciae* results in a mutant that can induce nodules on peas, but with greatly reduced level of symbiotic nitrogen fixation. Electron microscopy of the nodules revealed that infection and release of the *glmS* mutant from infection threads was normal. However, the subsequent development of bacteroids was abnormal; bacteroids in the mature zone of the nodule were much larger than controls, were abnormally shaped and highly vacuolated, and underwent rapid senescence. It is proposed that expression of *nodM* (also encoding a glucosamine synthase), present on the symbiotic plasmid, enabled the mutant to grow in the rhizosphere and within infection threads, but when the bacteria were released from infection threads, the *nod* genes (including *nodM*) were no longer expressed, resulting in glucosamine limitation of the bacteroids. Similarly, glucosamine limitation in free-living cultures caused a significant reduction in the amount of cell wall lipopolysaccharide and in qualitative changes to the lipopolysaccharide, as revealed by probing with monoclonal antibodies targeted against lipopolysaccharide epitopes.

Additional keyword: *Pisum sativum*.

Some rhizobial strains possess two glucosamine synthases (Marie *et al.* 1992; Baev *et al.* 1991). One of these, GlmS, appears to be a chromosomally encoded enzyme involved in the synthesis of glucosamine precursors required for normal growth (Marie *et al.* 1992). In particular, glucosamine residues constitute major components of the lipopolysaccharide of the rhizobial cell wall (Carlson *et al.* 1992). A strain of *Rhizobium leguminosarum* biovar *viciae* mutated in the *glmS* gene was found to be auxotrophic for glucosamine (Marie *et al.* 1992).

One of the glucosamine synthases that is present in *R. l.* bv. *viciae* (Marie *et al.* 1992) and *R. meliloti* (Baev *et al.* 1991) is encoded by the nodulation gene (*nodM*) and is induced by flavonoids secreted from legume roots. The *nodM* gene product supplements the supply of glucosamine precursors required for the formation of the lipo-oligosaccharide nodulation factors (Baev *et al.* 1991, 1992; Marie *et al.* 1992). These Nod factors all consist of a backbone of *N*-acetyl glucosamine residues that is decorated with a variety of substituents (Dénarié *et al.* 1992; Spaink 1992) that determine the range of legumes nodulated by any given rhizobial strain.

The two glucosamine synthases are functionally homologous, and the *glmS* mutant of *R. l.* bv. *viciae* can be restored to prototrophy by induction of the *nodM* gene by flavonoids (Marie *et al.* 1992). Conversely, mutation of *nodM* does not block nodulation, because the *glmS* gene product provides a sufficient level of glucosamine precursors to allow synthesis of the Nod factors (Marie *et al.* 1992; Baev *et al.* 1992). However, whereas mutation of *nodM* in *R. l.* bv. *viciae* had relatively little effect on the level of nodulation of peas or vetch (Surin and Downie, 1988), mutation of *nodM* in *R. meliloti* significantly reduced the nodulation of *Medicago sativa* (Baev *et al.* 1992). This difference in the effects of mutation of *nodM* could be explained if the level of synthesis of glucosamine precursors by the "housekeeping" glucosamine synthase (GlmS) in *R. l.* bv. *viciae* is considerably higher than that found with *R. meliloti*.

In addition to forming the appropriate lipo-oligosaccharide Nod factor to establish an effective symbiosis, the rhizobia must make the appropriate surface polysaccharides. Mutations affecting the formation of acidic exopolysaccharide can block normal infection and normal growth of the rhizobia within their host legumes (Leigh and Coplin 1992). Similarly, mutations affecting the formation of a normal lipopolysaccharide can severely affect the development of nitrogen-fixing nodules (Noel *et al.* 1986; Perotto *et al.* 1993). The ineffective nodules formed on peas by lipopolysaccharide mutants of *R. l.* bv. *viciae* were found to have relatively few infected cells, and the cell invasion was often associated with a plant defense reaction (Priefer 1989; Perotto *et al.* 1994). A mutant of *R. l.* bv. *viciae* lacking the *glmS* gene but carrying a normal *nodM* gene was found to nodulate peas and vetch, but the nodules had a low level of nitrogen fixation (Marie *et al.* 1992). Since this mutant is affected in the synthesis of glucosamine, the ineffective symbiosis might be due to abnormal infection or abnormal bacteroid development caused by a defect in the synthesis of the lipo-oligosaccharide Nod factor and/or the cell wall lipopolysaccharide. Here we have analyzed the ultrastructure of nodules formed by the mutant and examined the effects of the *glmS* mutation on the formation of the lipopolysaccharide.

RESULTS

Ultrastructure of pea nodules induced by a *glmS* mutant.

When *R. l.* bv. *viciae* strain A220 (*glmS2*::Tn*5lacZ*) was inoculated onto pea seedlings, the first nodules appeared at

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the same time as those induced by the control strain 8401/pRL1JI. However, over a 3-wk period the number of nodules formed by the mutant strain A220 was 60% higher than the control, and the nodules formed by A220 never developed the normal pink color typical of nitrogen-fixing nodules. Despite the increased nodule number, measurements of acetylene reduction on whole root systems of typical plants indicated that nitrogen fixation was only 5–20% of normal. This reduced level of nitrogen fixation was also evident from the poor growth of the plants in the nitrogen-free medium.

Young nodules from peas inoculated with the mutant (A220) and control (8401/pRL1JI) were embedded in resin and sectioned longitudinally for electron microscopy. A series of electron micrographs taken from one section of a typical nodule induced by strain A220 are shown in Figure 1. The longitudinal section of the nodule of the pea nodules enabled us to observe the temporal development of bacteroids from the early infection zone at the tip of the nodule to the mature zone close to the root. The large micrograph in Figure 1 shows a montage of an entire nodule section in which it is

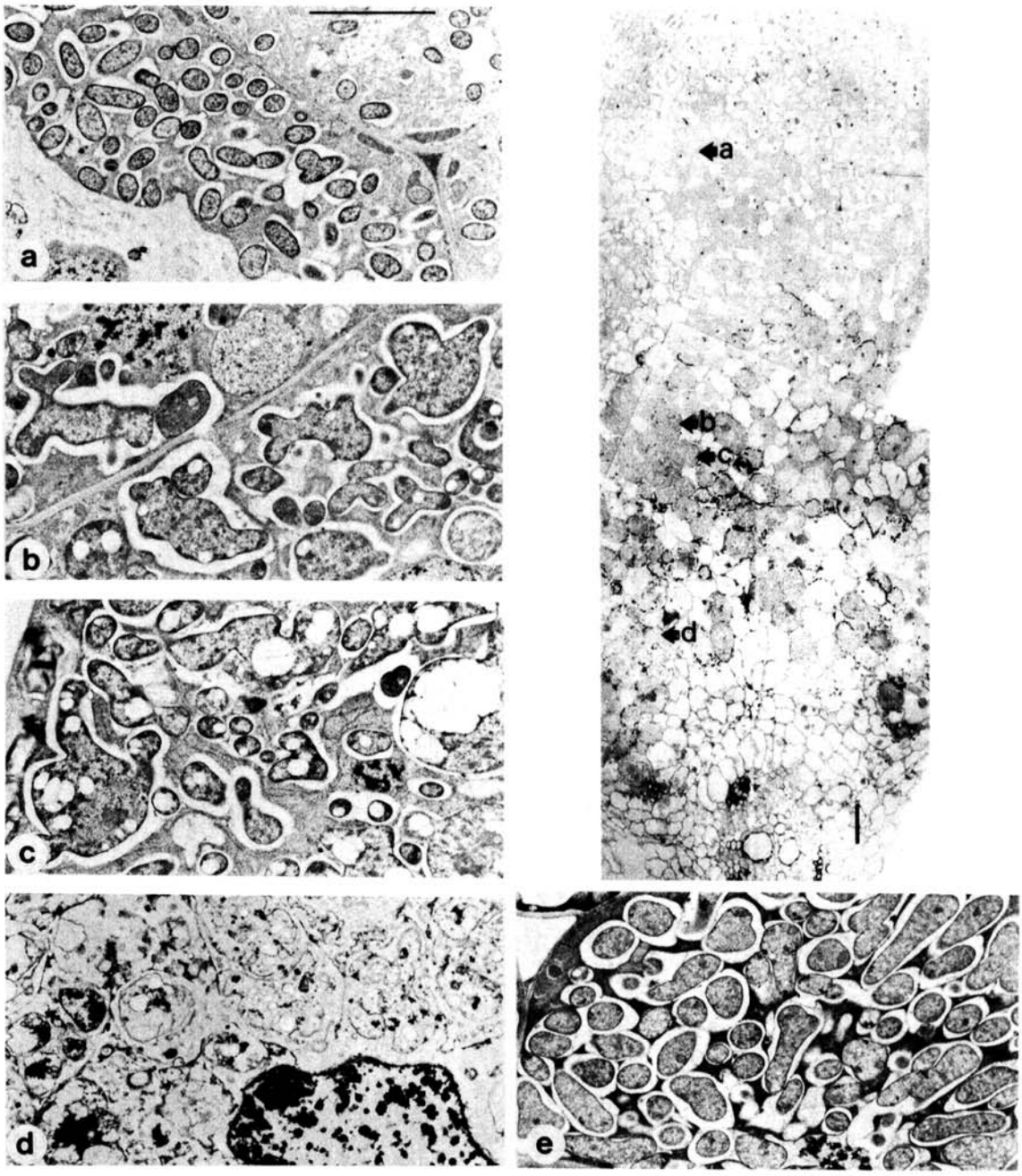

Fig. 1. Electron micrographs of a nodule induced by A220 (*glmS*). The large panel shows a longitudinal section of a typical 5-day-old nodule induced by the glucosamine auxotroph A220. The bar corresponds to 50 μm. The lettered arrows on the large panel point to the areas shown in the enlargements: **A,** early infected zone; **B and C,** mature zone; and **D,** zone of premature senescence. The bar A corresponds to 5 μm and the same magnification is used in B–E. For comparison, **E** shows a section of a micrograph from the mature zone of a nodule (equivalent to the areas covered by B and C) induced by the control strain 8401/pRL1JI.

possible to see early senescence within the mature zone even though the nodule is only 5 days old; senescence is not normally seen in nodules less than 10 days old. Panels A–D in Figure 1 are enlargements of specific regions as indicated on the whole section. After normal infection and bacteroid release the bacteroids in the young infected zone appeared normal (Fig. 1A), but they did not develop normally. Within the mature zone (Fig. 1B, C) the bacteroids were larger than normal, atypical shaped, and were either highly vacuolated or contained poly-β-hydroxybutyric acid inclusion granules. For comparison, bacteroids from the mature zone of a nodule of similar age induced by the control strain are shown in Figure 1E. Figure 1D shows the degradation of bacteroids in a prematurely senescent cell.

Analysis of the lipopolysaccharide from a glucosamine auxotroph.

The abnormal development of bacteroids was similar to that seen with LPS mutants of *R. l.* bv. *viciae* (Perotto *et al.* 1994) and may be due to a lack of glucosamine precursors required for LPS biogenesis in bacteroids. To analyze the effects of glucosamine limitation during free-living growth we analyzed the LPS in the glucosamine auxotroph A219 (*glmS2*::Tn*5*) which lacks the *nodM* gene because the symbiotic plasmid is deleted. The total LPS content of mutant A219 grown under glucosamine limitation was compared with that of the control (8401) by silver staining of the LPS fractionated on a polyacrylamide gel. As shown (Fig. 2), LPS can be subdivided into two major fractions: LPS I, which contains lipid A core oligosaccharide and an O-antigen polysaccharide chain containing different numbers of sugar residues, and LPS II which lacks the O-antigen domain (Carlson *et al.* 1992). The *glmS* mutant A219 has a reduced level of LPS II and lacks many of the high molecular weight bands typically found in the LPS I region (Fig. 2). This abnormal pattern is restored to normal by the *nodM* gene expressed from a vector promoter on pIJ1852 (Fig. 2) or by the cloned *glmS* gene on pIJ1910 (not shown).

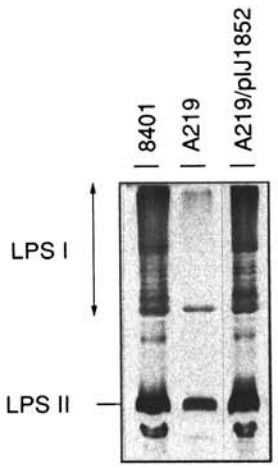

Fig. 2. Effects of glucosamine limitation on LPS. Samples were prepared from the control strain 8401, the glucosamine auxotroph A219, and A219 carrying nodM on pIJ1852, separated on a polyacrylamide gel and stained with periodate and silver stain. The loading was equivalent to 10 μg of cellular protein for each sample. The LPS I and LPS II fractions are indicated. Strain A219 was limited in its growth by the level of glucosamine present and shows a significantly reduced level of LPS. A normal pattern of LPS was seen when A219 was grown in the presence of sufficient glucosamine (300 μg/ml) (not shown).

It appears from Figure 2 that there is both a quantitative and a qualitative change in the LPS formed by A219 grown under glucosamine limitation. It is possible to monitor changes in the LPS immunologically, since a series of monoclonal antibodies have been identified which recognize different LPS epitopes (Sindhu *et al.* 1990; Wood *et al.* 1989). To visualize possible qualitative changes, the LPS from A219 grown under glucosamine limitation was probed with two monoclonal antibodies MASM-1 which recognizes the KDO component of the LPS and JIM32 which primarily recognizes the LPS II domain but also recognizes some LPS I bands (Fig. 3). In this gel the loadings of the samples were adjusted to give equivalent amounts of LPS by increasing the amount of sample loaded from strain A219. The LPS from A219 contains a component that comigrates with LPS II but when probed with JIM32 it was found to contain a number of novel bands and lack several of the typical LPS bands (Fig. 3C). Similarly, probing with MASM-1 revealed novel bands migrating between the LPS I and LPS II fractions (Fig. 3B). When the *nodM* gene was introduced on pIJ1852, the normal pattern of bands was restored (Fig. 3B, C) and a similar result was seen by introducing the *glmS* gene on pIJ1910 (data not shown).

Some of the monoclonal antibodies tested revealed that the pattern of LPS-staining bands found in A219 is more typical of that found in bacteroids than in cells grown in free-living cultures. This is illustrated with JIM21 and JIM22. Each of these monoclonal antibodies recognizes high-molecular weight bands present in the control 8401 (Fig. 4B, C) but absent from both bacteroids and cells of A219 grown under glucosamine limitation (Fig. 4B, C).

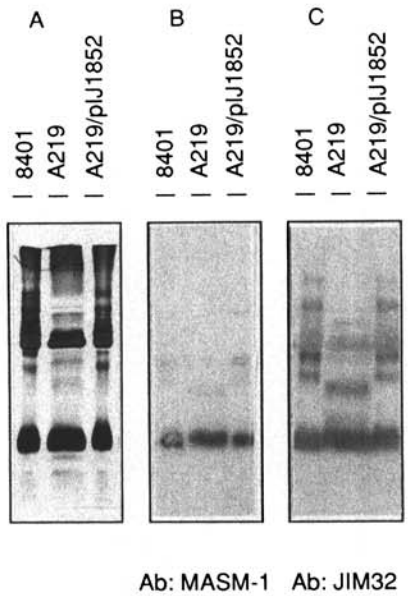

Fig. 3. Immunostaining of LPS of the glucosamine auxotroph. After separation on a polyacrylamide gel as described in Figure 2, the gel was silver stained **(A)** or immunostained using MASM-1 **(B)**, which recognizes the ketodeoxyoctonate of the LPS or with JIM32 **(C)**, which primarily recognizes LPS II components but also recognizes LPS I. Samples 8401 and A219/pIJ1852 were loaded at an equivalent of 10 μg protein per lane while the glucosamine limited A219 was loaded at an equivalent of 50 μg protein per lane.

We have identified a second-site revertant of the glucosamine auxotroph A220, in which the reversion is most probably caused by a mutation in the promoter of *nodM* to give prototrophy as a result of flavonoid-independent *nodM* expression (Marie 1992). This revertant (A300) grows to the same cell density as the control strain, but in the absence of glucosamine its growth rate is lower than the control (the doubling time was increased from 2.5–4 hr in TY medium). Although growth of this strain is limited by glucosamine (normal growth was restored by addition of glucosamine), it formed normal nitrogen-fixing nodules. However, as shown (Fig. 4), this mutant failed to produce the high molecular weight LPS material that is recognized by the monoclonal antibodies JIM21 and JIM22. The absence of these high molecular weight LPS I components in strains limited for growth by glucosamine, and in bacteroids may indicate that normal bacteroids have a limited capacity to synthesize glucosamine in nodules.

DISCUSSION

The principal role for the two glucosamine synthases GlmS and NodM in rhizobial strains is the formation of glucosamine precursors used in the synthesis of lipooligosaccharide Nod factors (Baev *et al.* 1991, 1992; Marie *et al.* 1992) and the LPS component of the rhizobial cell wall (Carlson *et al.* 1992). The glucosamine auxotrophy of the *glmS* mutant of *R. l.* bv. *viciae* can be suppressed by growing the bacteria in the presence of flavonoids that induce the expression of the *nod* genes (Marie *et al.* 1992). It is this induction

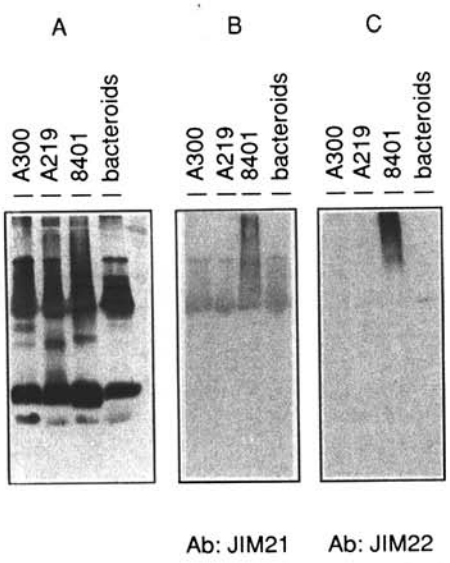

Fig. 4. LPS I components are absent from glucosamine limited cells and from bacteroids. Samples were silver stained **(A)** or immunostained with JIM21 **(B)** or JIM22 **(C)** following electrophoresis. Stain A300 contains a second-site mutation that partially suppresses the glucosamine auxotrophy caused by the *glmS2*::Tn*5lacZ* mutation. The high molecular weight LPS I epitopes recognized in 8401 by JIM21 and JIM22 are absent from the glucosamine-limited A219, from A300 (which was grown in the absence of glucosamine) and from bacteroids. JIM22 only weakly recognized the major low molecular weight LPS I component which is present as a single band. The bacteroids were isolated from 12-day-old nodules from peas inoculated with 8401/pRL1JI as described by Brewin *et al.* (1985). The sample loadings were adjusted to give approximately equal amounts of LPS as judged by the silver staining in A.

that enables the *glmS* mutant A220 to grow in the rhizosphere and in the infection threads. Glucosamine is unavailable from legume roots (Feingold and Avigad 1980), and a double mutant of *R. l.* bv. *viciae* lacking both *nodM* and *glmS* does not grow in the rhizosphere or infect peas or vetch (Marie *et al.* 1992).

The relatively normal early development that occurs in pea nodules induced by the *glmS* auxotroph A220 indicates that the *nod* genes are expressed throughout the infection process, confirming the observations of Sharma and Signer (1990) that *nod* gene fusions are expressed within infection threads. After the *glmS* mutant bacteria are released from the infection threads the initial development of bacteroids is normal. However, within the mature zone of the nodule, the bacteria become enlarged, vacuolated, and irregular in shape. This probably results from a deficiency in glucosamine caused by a cessation of *nod* gene expression. This correlates well with the observation of Schlaman *et al.* (1991). Using *in situ* RNA hybridizations, they found that there were *nod* gene transcripts in the invasion zone, but that *nod* gene expression was very low in the early symbiotic zone and absent in the mature zone of nodules. They concluded that there is a repressor that inhibits *nod* gene expression by bacteroids. Schlaman *et al.* (1992) identified a protein in bacteroids (but not in free-living cells) that bound to *nodA-nodD* promoter region and speculated that it might be such a repressor.

Baev *et al.* (1992) reported that a mutant *R. meliloti* lacking *nodM* was delayed in nodulation and that the bacteroids showed altered development resulting in nodules with lowered levels of nitrogen fixation. This also implies that *nodM* is expressed by *R. meliloti* nodules and that the level of glucosamine formed by the GlmS enzyme of *R. meliloti* is a limiting factor during bacteroid growth. This appears not to be the case in *R. l.* bv. *viciae* since mutation of *nodM* had no effect on nodulation or on nitrogen fixation (Surin and Downie 1988; Marie *et al.* 1992). This difference may reflect a difference in the levels of expression of the *glmS* gene in the two different *Rhizobium* strains.

It is clear that in the absence of *glmS* expression the level of LPS synthesis by *R. l.* bv. *viciae* is greatly reduced, but can be restored by expressing either *nodM* or *glmS*. During glucosamine limitation the structure of the LPS also appears to be different from normal, since the size fractionation of LPS bands following silver staining or probing with monoclonal antibodies revealed the absence of several components and the appearance of novel bands. The development of this abnormal LPS may contribute to the rapid senescence of the rhizobia in the nodules. Indeed the changes in bacteroid size and shape observed with the *glmS* auxotroph are remarkably similar to those found with some Fix$^-$ mutants of *R. l.* bv. *viciae* directly affected in LPS synthesis (Perotto *et al.* 1994).

During normal bacteroid development there are substantial changes to the LPS of *R. l.* bv. *viciae* and these can be followed using epitope-specific monoclonal antibodies (Sindhu *et al.* 1990; Wood *et al.* 1989). One of these changes is the disappearance of the high molecular weight LPS I components recognized by JIM21 and JIM22. As shown here, these components are also lost under conditions of glucosamine limitation during free-living culture. One explanation for their loss in bacteroids may therefore be that glucosamine availability may be limiting for bacteroids. This could be caused

by a low availability of glutamine or of fructose-6-phosphate, both of which are required for the synthesis of glucosamine. If glucosamine is indeed limiting, then LPS biosynthesis would be reduced. One consequence of this might be that cell division might be affected, possibly resulting in the observed increase in bacteroid volume compared with free-living bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth.

R. leguminosarum strains 8401, lacking a symbiotic plasmid, and 8401/pRL1JI, carrying the bv. *viciae* symbiotic plasmid pRL1JI, were described by Downie *et al.* (1983). A219 and A220 are derivatives of 8401 and 8401/pRL1JI, respectively, carrying the *glmS2*::Tn*5lacZ* allele (Marie *et al.* 1992). pIJ1852 carries the cloned *nodM* gene expressed from a vector promoter (Marie *et al.* 1992) and pIJ1910 carries the cloned *glmS* gene expressed from its own promoter.

R. leguminosarum strains were grown in TY medium (Beringer 1974) containing the appropriate antibiotics to maintain selection for plasmids. For the preparation of glucosamine-limited cultures, cells were first grown for 48 hr at 30° C in TY medium containing 200 μg ml^{-1} glucosamine and then for 24 hr in TY medium lacking glucosamine. Normal growth required 300 μg ml^{-1} glucosamine to be added.

Analysis of nodulation, acetylene reduction, and preparation of nodules for microscopy.

Peas, cultivar Wisconsin Perfection, were inoculated with strain A220 which had been grown on TY agar medium containing glucosamine and grown as described by Beynon *et al.* (1980). The numbers of nodules found were scored on alternate days and compared with control plants inoculated with 8401/pRL1JI. Twelve matched plants were scored for the rate of nodulation and measurements of acetylene reduction were made using intact root systems of peas harvested 21 days after inoculation as described by Beynon *et al.* (1980). Bacteroids were isolated from 12-day-old nodules from peas inoculated with 8401/pRL1JI following the procedure of Brewin *et al.* (1985), involving fractionation by sucrose density gradient centrifugation.

For microscopy, 5-day-old nodules were excised from plants 15 days after inoculation of the plants with A220 or 8401/pRL1JI. The nodules were embedded and prepared for electron microscopy as described by Beringer *et al.* (1977) using gluteraldehyde and osmium for fixation and LR white resin. Two nodules from each of four plants were analyzed and the sections shown are typical of most of the nodules.

Analysis of lipopolysaccharide.

Samples for gel separation were prepared with proteinase K treatment as described by Sindhu *et al.* (1990), and the SDS polyacrylamide gel separation system was as described by Kannenberg and Brewin (1989). The protein content of samples was estimated, prior to the addition of protease, using the Bio-Rad protein assay kit. Gels were stained for carbohydrates using periodate and silver staining as described by Wood *et al.* (1989) or transferred to nitrocellulose, and LPS epitopes were visualized using monoclonal antibodies as described by Kannenberg *et al.* (1992). MASM-1 is a mouse monoclonal antibody with specificity for the ketodeoxyoctonate of the LPS molecules (Lind *et al.* 1985, Wood *et al.* 1989). The rat monoclonal antibodies JIM21 and JIM22 react with LPS epitopes from *R. leguminosarum* 8401 (Sindhu *et al.* 1990; E. Kannenberg, personal communication). JIM32 reacts with LPS I and LPS II components (M. Lucas and N. Brewin, personal communication).

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LITERATURE CITED

Baev, N., Endre, G., Petrovics, G., Banfalvi, Z., and Kondorosi, A. 1991. Six nodulation genes of *nod* box locus 4 in *Rhizobium meliloti* are involved in nodulation signal production: *nodM* codes for D-glucosamine synthetase. Mol. Gen. Genet. 228:113-124.

Baev, N., Schultze, M., Barlier, I., Ha, D. C., Virelizier, H., Kondorosi, A., and Kondorosi, E. 1992. *Rhizobium nodM* and *nodN* genes are common *nod* genes: *nodM* encodes functions for efficiency of nod signal production and bacteroid maturation. J. Bacteriol. 174:7555-7565.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-198.

Beringer, J. E., Johnston, A. W. B., and Wells, B. 1977. The isolation of conditional ineffective mutants of *Rhizobium leguminosarum*. J. Gen. Microbiol. 98:339-343.

Beynon, J. L., Beringer, J. E., and Johnston, A. W. B. 1980. Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. 120:421-429.

Brewin, N. J., Robertson, J. G., Wood, E. A., Wells, B., Larkins, A. P., Galfrè, G., and Butcher, G. W. 1985. Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membrane and Golgi bodies. EMBO J. 4:605-611.

Carlson, R. W., Bhat, U. R., and Reuhs, B. 1992. *Rhizobium* lipopolysaccharides: Their structure and evidence for their importance in the nitrogen-fixing symbiotic infection of their host legumes. Pages 33-44 in: Plant Biotechnology and Development. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.

Dénarié, J., Debelle, F., and Rosenberg, C. 1992. Signalling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531.

Downie, J. A., Ma, Q-S., Knight, C. D., Hombrecher, G., and Johnston, A. W. B. 1983. Cloning of the symbiotic region of *Rhizobium leguminosarum*: The nodulation genes are between the nitrogenase genes and the *nifA*-like gene. EMBO J. 2:947-952.

Feingold, D. S., and Avigad, G. 1980. Sugar nucleotide transformations in plants. Pages 101-170 in: The Biochemistry of Plants. Vol. 3, Carbohydrates: Structure and Function. J. Preiss, ed. Academic Press, New York.

Kannenberg, E. L., and Brewin, N. J. 1989. Expression of a cell surface antigen from *Rhizobium leguminosarum* 3841 is regulated by oxygen and pH. J. Bacteriol. 171:4543-4548.

Kannenberg, E. L., Rathbun, E. A., and Brewin, N. J. 1992. Molecular dissection of structure and function in the lipopolysaccharide of *Rhizobium leguminosarum* strain 3841 using monoclonal antibodies and genetic analysis. Mol. Microbiol. 6:2477-2487.

Leigh, J. A., and Coplin, D. L. 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. 46:307-346.

Lind, S. M., Carlin, N. I. A., and Lindberg, A. A. 1985. Production and characterization of KDO-specific monoclonal antibodies, recognizing lipopolysaccharides from heptoseless mutant of *Salmonella*. FEMS Microbiol. Lett. 28:45-49.

Marie, C. 1992. Roles of two *Rhizobium leguminosarum* glucosamine synthases in symbiosis. Ph.D. thesis. University of East Anglia, Norwich, U.K.

Marie, C., Barny, M.-A., and Downie, J. A. 1992. *Rhizobium leguminosarum* has two glucosamine synthases, GlmS and NodM, required for nodulation and development of nitrogen-fixing nodules. Mol. Microbiol. 6:843-851.

Noel, K. D., VandenBosch, K. A., and Kulpaca, B. 1986. Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. J. Bacteriol. 168:1392-1401.

Perotto, S., Brewin, N. J., and Kannenberg, E. L. 1994. Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. Mol. Plant-Microbe Interact. 7:99-112.

Priefer, U. B. 1989. Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. J. Bacteriol. 171:6161-6168.

Schlaman, H. R. M., Horvath, B., Vijgenboom, E., Okker, R. J. H., and Lugtenberg, B. J. J. 1991. Suppression of nodulation gene expression in bacteroids of *Rhizobium leguminosarum* biovar *viciae*. J. Bacteriol. 173:4277-4287.

Schlaman, H. R. M., Lugtenberg, B. J. J., and Okker, R. J. H. 1992. The NodD protein does not bind to promoters of inducible nodulation genes in extracts of bacteroids of *Rhizobium leguminosarum* biovar *viciae*. J. Bacteriol. 174:6109-6116.

Sharma, S. B., and Signer, E. R. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-*gusA*. Genes Development 4:344-356.

Sindhu, S. S., Brewin, N. J., and Kannenberg, E. L. 1990. Immunochemical analysis of lipopolysaccharides from free-living and endosymbiotic forms of *Rhizobium leguminosarum*. J. Bacteriol. 172:1804-1813.

Spaink, H. P. 1992. Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. 20:977-986.

Surin, B. P., and Downie, J. A. 1988. Chracterization of the *Rhizobium leguminosarum* genes *nodLMN* involved in efficient host specific nodulation. Mol. Microbiol. 2:173-183.

Wood, E. A., Butcher, G. W., Brewin, N. J., and Kannenberg, E. L. 1989. Genetic derepression of a developmentally regulated lipopolysaccharide antigen from *Rhizobium leguminosarum* 3841. J. Bacteriol. 171:4549-4555.