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CONTENTS

Contents continued on next page

On the cover: Stem segments of *Sesbania rostrata* plants with nodules produced by *Azorhizobium caulinodans*. For the article by Pawlowski *et al.*, see page 35. (Photographs by Margaret Kalda, MPI, Cologne)

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RESEARCH NOTES

ExoB Mutants of *Bradyrhizobium japonicum* with Reduced Competitiveness for Nodulation of *Glycine max*

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Exopolysaccharide (EPS) mutants of *Bradyrhizobium japonicum* defective within a DNA region homologous to the *Rhizobium meliloti exoB* gene were constructed. Using an interspecies complementation approach, two overlapping cosmid clones of *B. japonicum* DNA were isolated. A 9.1-kb *Eco*RI subclone common to both cosmids was found to restore the ability of the *exoB* mutant *R. meliloti* H36, to induce effective nodules on alfalfa plants, and to form fluorescent colonies on agar media containing cellufluor white. Km^r deletion as well as insertion derivatives of this fragment were introduced into *B. japonicum* 110*spc*4 by marker exchange. The resulting deletion mutants ΔP5, ΔP6 (4.5 kb each), and ΔP22 (2.1 kb) were designated *exoB* mutants, because they lacked UDP-glucose 4′-epimerase activity, and the deleted regions hybridized with an *exoB* DNA probe of *R. meliloti*. The mutants had a nonmucoid colony morphology. In contrast to the wild-type EPS, no galactose could be detected in the residual EPS produced by the mutant strains, indicating an altered EPS composition. The mutant strains exhibited a wild-type lipopolysaccharide pattern on polyacrylamide gels. Although the mutants induced effective nodules on soybean, the early stages of the symbiotic interaction were disturbed. Nodulation was delayed by about 5 days, and the mutants exhibited a greatly reduced competitiveness. When the mutants ΔP5 or ΔP22 were coinoculated together with the parent strain 110 at similar titers, almost all of the nodules were occupied by the wild type. An at least 100-fold excess of ΔP5 or ΔP22 cells was necessary to obtain half of the nodules occupied by the mutant strains. This effect is not due to the presence of the *aph* gene in these mutants, as revealed by the unaffected competitiveness of the insertion mutant P29. The reduced competitiveness of *B. japonicum exo* mutants for nodulation of soybean clearly demonstrates that exopolysaccharides of *B. japonicum* carry out important functions during the early stages of the symbiotic interaction.

Additional keywords: infection, nodulation, symbiosis.

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As a consequence of the complex molecular communication process between rhizobia and leguminous host plants, the microsymbiont induces the formation of a new plant organ, the nitrogen-fixing root nodule. Rhizobial factors that are involved in this specific process are of major interest, and their analysis should eventually lead to a better understanding of plant-microbe interactions in general. Due to their exposed localization, rhizobial surface polysaccharides are predisposed to be such a factor. However, following current opinion, their significance seems to depend on the nodule type produced by the host plant. Various forms of nodules can be found that can be grouped into two principally different types. Determinate nodules are spherical, and meristematic activity of plant cells stops at a certain predetermined developmental stage. In contrast, indeterminate nodules are cylindrical and have a persistent meristem. It has been demonstrated that specific rhizobial exopolysaccharide (EPS) structures are necessary for the infection of indeterminate nodule-type legumes (e.g., *Leucaena, Medicago, Pisum, Trifolium,* and *Vicia* species) by rhizobia (Borthakur *et al.* 1986; Chakravorty *et al.* 1982; Diebold and Noel 1989; Hotter and Scott 1991; Leigh *et al.* 1987; Müller *et al.* 1988). EPS mutants of the corresponding microsymbionts *R. loti, R. meliloti,* or *R. leguminosarum* with reduced symbiotic capabilities have been described. A typical phenotype is the induction of uninfected nodulelike structures that are devoid of bacteroids. Infection threads are occasionally formed but abort prematurely, indicating that infection is blocked at an early stage of the interaction (for recent reviews see Brewin 1991; Kijne 1992).

Exopolysaccharide mutations that block infection of indeterminate nodule-type legumes do not abolish effective nodule development of the determinate nodule-type legumes *Phaseolus* or *Lotus* (Borthakur *et al.* 1986; Diebold and Noel 1989; Hotter and Scott 1991). It has therefore been concluded that EPS is not essential for the infection of determinate nodule-type legumes. For soybean, another determinate nodule-forming legume, the results obtained for a number of Tn*5*-induced exopolysaccharide mutants of *R. fredii* were interpreted as supportive of this view, since effective nodules are formed by most of these mutants (Kim *et al.* 1989; Ko and Gayda 1990). However, care should be taken because "not essential" does not necessarily mean "without function." Other symbiotic parameters in addition to the ability to reduce acetylene have to be analyzed to unravel these functions. A decade ago, the laboratory of W. D. Bauer described a set of spontaneous *B. japonicum* mutants with altered capsule synthesis (Law *et al.* 1982), all of which were able to nodulate soybean when applied at high inoculum titers. However, a careful analysis revealed that some of the mutants had reduced nodulation ability when inoculated at suboptimal titers. These results demonstrate that more sensitive assay systems have to be applied to reveal the symbiotic effects of EPS mutations in determinate symbioses. These observations point toward an influence of bradyrhizobial EPS on the symbiotic interaction with *Glycine* and encouraged us to start an investigation based on defined exopolysaccharide mutants of *B. japonicum* that were not available until now. Based on their different physical appearance, the extracellular polysaccharides of *B. japonicum* observed in broth culture have classically been subdivided into a capsular (CPS) and soluble (EPS) fraction. However, the analysis of their chemical composition did not reveal major differences (Mort and Bauer 1982). In the present paper, the term EPS refers to both EPS and CPS of *B. japonicum*.

Because of the similarities in the biosynthetic precursors of EPS and LPS, pleiotropic mutations affecting both polymers are often observed (Baghwat *et al.* 1991; Diebold and Noel 1989). Consequently, it is difficult to ascertain whether EPS or LPS are responsible for the symbiotic defect. Lipopolysaccharides are thought to be of major importance for the infection of determinate nodule-type legumes (Stacey *et al.* 1991). To exclude interference of LPS effects with our results, we constructed mutants of *B. japonicum* with specific defects in their EPS but not in their LPS production. In this work, we made use of the fact that EPS of *B. japonicum* 110 contains galactose, while the LPS does not (Puvanesarajah *et al.* 1987). Therefore, a specific defect in the biosynthesis of UDP-galactose should lead to an EPS-specific mutant. In *Rhizobium leguminosarum* and in *R. meliloti* UDP-galactose is formed from UDP-glucose by UDP-glucose 4′-epimerase (Canter Cremers *et al.* 1990), the product of the *exoB* gene (Buendia *et al.* 1991). Tn*5*-induced *exoB* mutants of *R. l.* bv. *viciae* synthesize a galactose-free exopolysaccharide (Canter Cremers *et al.* 1990). Assuming that similar pathways are operative in *B. japonicum*, the elimination of an *exoB*-like gene should be a suitable strategy to obtain a mutant with specific defects in EPS but not in LPS synthesis. Here we describe the construction of such mutants and show that they are specifically altered in EPS production. Their reduced symbiotic abilities on soybean are most pronounced under competitive conditions, indicating the importance of correct EPS structure in the early stages of the symbiotic interaction in determinate nodule-type legumes.

RESULTS

Isolation of *B. japonicum* DNA carrying a region homologous to the *R. meliloti exoB* gene.

Interspecies complementation was used to isolate an *exoB* homologous gene from *B. japonicum*. A genomic library of *B. japonicum* DNA was constructed based on the cosmid vector pVK100. The cosmids were introduced in *Rhizobium meliloti* H36, a deletion mutant lacking 400 bp of megaplasmid 2, including parts of the *exoB* gene (Buendia *et al.* 1991). In contrast to its parent strain *R. meliloti* 2011, this mutant is unable to induce effective nodules on alfalfa plants and its colonies do not show fluorescence on Cellufluor white containing agar media (cfw agar) when irradiated with UV light. The strains carrying a cosmid were analyzed for their capacity to form fluorescent colonies on cfw agar. Two overlapping cosmids could be isolated that restored fluorescence of the mutant strain. Both cosmids contained two *Pst*I fragments of 2.1 and 2.4 kb that hybridized weakly with an internal fragment of the *R. meliloti exoB* gene (not shown). A 9.1-kb *Eco*RI fragment containing both *Pst*I fragments was subcloned in pSUP102 resulting in plasmid pBJ1. This plasmid complemented *R. meliloti* H36 to form fluorescent colonies on cfw agar and to nodulate alfalfa effectively. The results of these hybridization and complementation analyses suggested that an *exoB* homologous gene is located on the 9.1-kb *Eco*RI fragment, most probably on the internal 4.5-kb *Pst*I region (Fig. 1). DNA sequence analysis supports this conclusion (unpublished data). The 9.1-kb *Eco*RI fragment was subjected to further analysis.

Construction of *B. japonicum exoB* mutants.

Because both internal 2.1- and 2.4-kb *Pst*I fragments of pBJ1 hybridized weakly with an internal fragment of the *R. meliloti exoB* gene, this 4.5-kb region was subjected to mutational analysis. Derivatives of pBJ1 were constructed either by replacing internal *Pst*I fragments with the kanamycin resistance cassette of pUC4K or by introduction of the cassette into internal *Pst*I sites. The corresponding mutants of *B. japonicum* 110*spc*4 were constructed by marker exchange mutagenesis. The genotypes of the resulting strains are shown in Figure 1. In Figure 2, an analysis of total DNA of the mutant strains is shown, confirming the nature of the mutations. Strains *B. japonicum* P9, P23, and P29 carry insertions, while in strains ΔP5, ΔP6, and ΔP22 *Pst*I fragments of different sizes are deleted (Fig. 1).

The deletion mutants lack UDP-glucose 4′-epimerase.

Because the deleted regions putatively contain an *exoB* homologous gene coding for UDP-glucose 4′-epimerase, the mutants were tested for activity of this particular

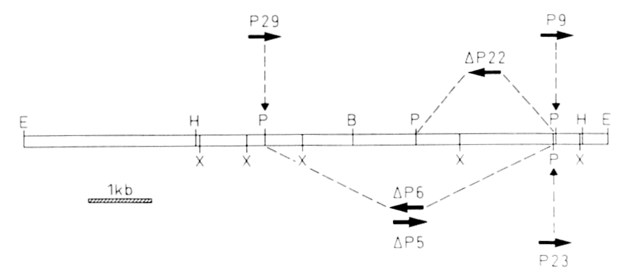

Fig. 1. Restriction map of the 9.1-kb *Eco*RI fragment of *Bradyrhizobium japonicum* 110*spc*4 DNA. The two internal *Pst*I fragments of 2.1 and 2.4 kb hybridized with a *exoB* specific probe of *R. meliloti*. The genotypes of the insertion mutants P9, P23, and P29 and of the deletion mutants ΔP5, ΔP6, and ΔP22 are indicated by the insertion points and orientation of the *aph* gene of pUC4K. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I; B, *Bam*HI.

enzyme. Whereas the wild type and the insertion mutants P9, P23, and P29 all showed comparable activities, no activity was found in extracts of deletion mutants ΔP5, ΔP6, or ΔP22 (Table 1). This finding supports the conclusion that the deletion mutants are impaired in the *exoB* gene. Southern hybridization experiments (e.g., Fig. 2) revealed only one hybridizing genomic 9.1-kb *Eco*RI fragment, when pBJ1 was used as the probe. On the basis of these genetic and biochemical data it is unlikely, that a second, redundant *exoB* gene is present in *B. japonicum*. The mutants ΔP5, ΔP6, and ΔP22 grew well on minimal media with galactose, succinate, or xylose as sole carbon source, indicating that the deleted region does not carry genes for general metabolism or housekeeping genes. Only specific functions involved in EPS synthesis appear to be impaired. The ability of *exoB* mutants to grow on galactose as the sole carbon source implies that UDP-glucose 4′-epimerase of *B. japonicum* is not required for catabolism of galactose. Similar observations were made for the enzyme from *R. meliloti* (Buendia *et al.* 1991) and *R. leguminosarum* (Canter Cremers *et al.* 1990).

Table 1. UDP-glucose 4′-epimerase activity in mutants of *Bradyrhizobium japonicum*

Strain	Epimerase activity[a]
B. japonicum	
110*spc*4	67
P9	61
P23	59
P29	70
ΔP5	0
ΔP6	0
ΔP22	0

[a] Activity of UDP-glucose 4′-epimerase is expressed as nmol × min^{-1} × mg $protein^{-1}$.

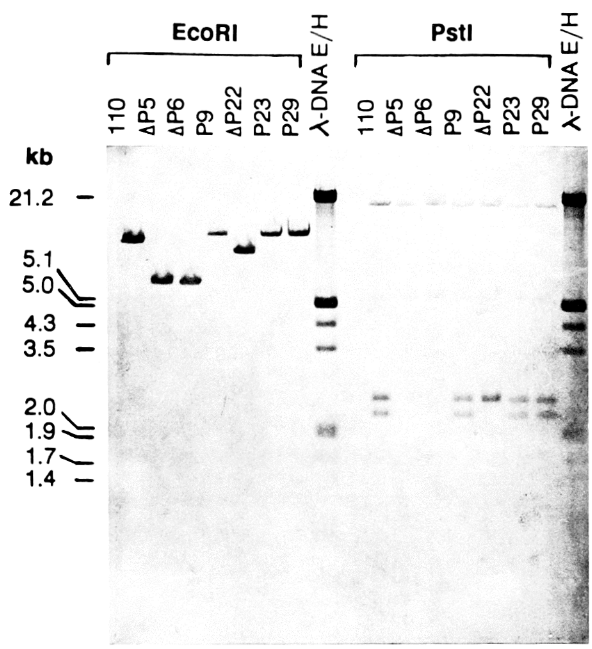

Fig. 2. Southern blot of *Eco*RI- and *Pst*I-digested genomic DNA of *Bradyrhizobium japonicum* hybridized with digoxigenin-labeled pBJ1, a pSUP102 derivative containing the 9.1-kb *Eco*RI fragment of *B. japonicum* DNA depicted in Figure 1. The fragment size of *Eco*RI/*Hin*dIII-digested lambda DNA is indicated.

The *exoB* mutants are altered in their EPS but not LPS structure.

B. japonicum 110*spc*4 forms mucoid colonies on agar media. When grown on 20E supplemented with Congo red, the thick layers of EPS do not accumulate the pigment, resulting in a white to faint red colony color. In contrast, the deletion mutants ΔP5, ΔP6, and ΔP22 form small, nonmucoid, red colonies (Fig. 3). The altered colony morphology indicates that these mutants are impaired in EPS production. The amount of EPS produced by the mutant during growth to an early stationary phase in liquid medium (SMM) was found to be about half of that produced by the wild type. An analysis of EPS composition by GC-MS revealed that, unlike the wild-type and insertion mutants, the EPS of all of the deletion mutants lacked galactose. Furthermore, an enzyme-dependent assay for galactose failed to detect galactose in the EPS of the deletion mutants. Because galactose is a constituent of the wild-type EPS pentasaccharide unit (Mort and Bauer 1982), these findings indicate that the mutant EPS must have an altered structure.

In contrast, LPS analysis by polyacrylamide gel electrophoresis did not show any differences between the wild-type and mutant LPS (Fig. 4). This is as expected, since LPS of *B. japonicum* 110 does not contain galactose (Puvanesarajah *et al.* 1987) and therefore should not be influenced by a mutation of an *exoB*-like gene.

The *exoB* mutants form effective nodules on soybean, but exhibit a strongly reduced competitiveness.

Soybean nodules induced by the different mutants all showed similar acetylene reduction rates per milligram nodule fresh weight 28 days after infection. This was determined by testing root sections carrying three to four well-developed nodules. To confirm that the observed fixation could be attributed to the mutants, the identity

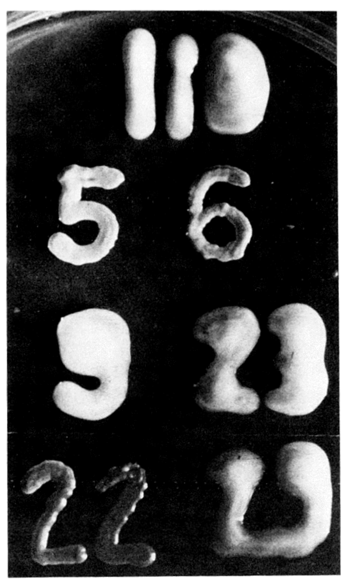

Fig. 3. Colony morphology of *Bradyrhizobium japonicum* 110*spc*4 and derivatives grown on 20E agar supplemented with Congo red. The nonmucoid colony surface of the mutants ΔP5 (5), ΔP6 (6), and ΔP22 (22) in contrast to the insertion mutants P9, P23, P29, and the wild type (110) is apparent.

of the occupying strains of each nodule was analyzed by testing for antibiotic resistance and colony morphology. In addition, total DNA from reisolated strains was extracted, and Southern hybridization experiments confirmed that no genetic alterations had occured, with respect to the DNA region under study, during passage through the nodule (not shown). Hence, it was demonstrated that *exoB* mutants are in principle equally capable of infecting the host plant and establishing an effective symbiosis as the wild type.

However, when analyzed in more detail, interesting differences were observed concerning the early stages of the symbiotic interaction. Nodules of plants infected with the deletion mutants appeared with a delay of approximately 5 days compared to the wild type (Fig. 5). This delay in nodule appearance is an indication of an impaired interaction between the symbiotic partners during the early stages of infection. The delay in nodulation is also reflected by the position of the nodules on the root system. The primary root of plants infected with the deletion mutants is almost free of nodules. This effect was quantitatively analyzed in growth pouches. The deletion mutants induce significantly reduced numbers of nodules above the root tip mark in comparison to the wild-type or the insertion mutant P29 (Fig. 6). This parameter has previously been shown to be a sensitive marker for nodulation ability of EPS mutants (Law *et al.* 1982).

The symbiotic defect of EPS mutants was even more apparent when their symbiotic performance was analyzed under competitive conditions. When coinoculated with the wild type, the *exoB* mutants exhibited a strongly reduced competitiveness (Table 2). Even when the mutants were applied in 10-fold excess, almost all nodules were formed by the wild-type strain. A 100-fold excess of mutant bacteria was necessary to obtain half of the nodules occupied by the mutant. This effect was not due to the presence of the *aph* gene as demonstrated by the unaffected competitiveness of P29, carrying this gene as an insertion (Fig. 1). It has already been shown that even the presence of the transposon Tn*5* in *B. japonicum* 110 conferring drug resistance to kanamycin and streptomycin does not influence per se the competitivity of this strain (Bhagwat *et al.* 1991; Hahn and Studer 1986).

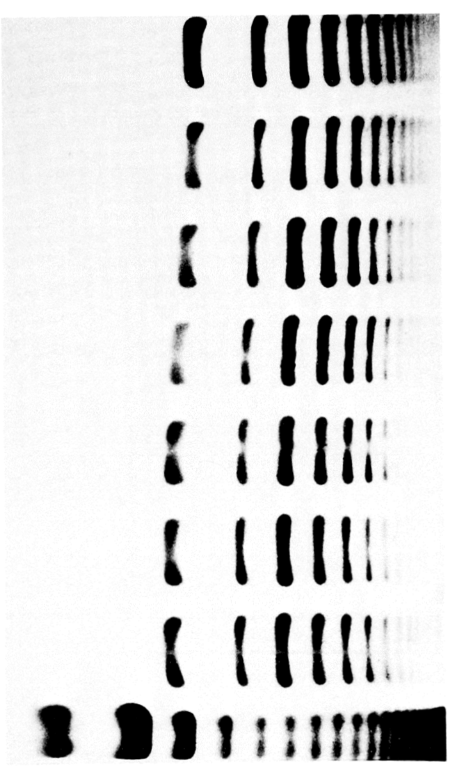

Fig. 4. Desoxycholate-polyacrylamide gel electrophoresis of *Bradyrhizobium japonicum* 110*spc*4 and derivatives. LPS of *Salmonella minnesota* (*S. min.*) was used as reference.

DISCUSSION

The aim of the present work was to evaluate the significance of exopolysaccharide produced by *B. japonicum* for the symbiotic interaction with *G. max*. This was accom-

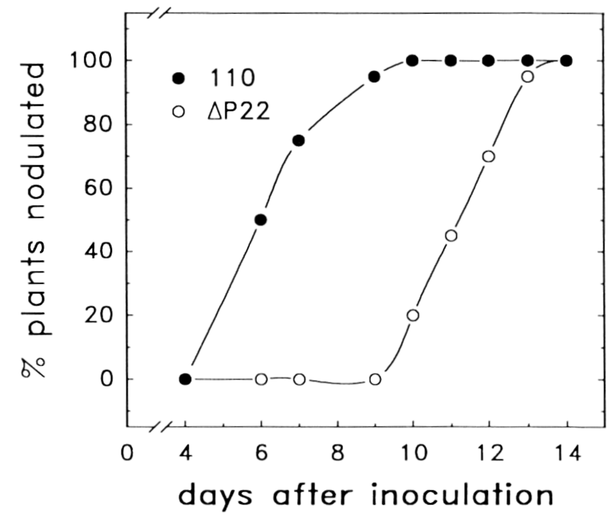

Fig. 5. Kinetic of nodule appearance on *Glycine max* 'Preston' infected by *Bradyrhizobium japonicum* 110*spc*4 and the deletion mutant ΔP22. Bacteria were grown in SMM and inoculated at 10^5 cfu/plant. Data are from 20 plants per strain grown in growth pouches. ΔP5 gave a kinetic almost similar to ΔP22 (not shown).

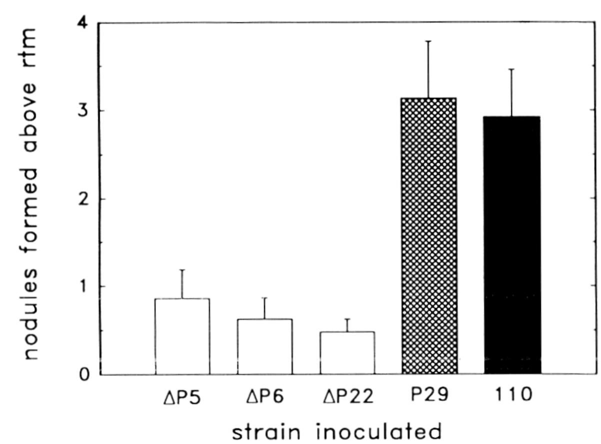

Fig. 6. Number of nodules above root tip mark/plant of *Glycine max* 'Preston' inoculated with *Bradyrhizobium japonicum* 110*spc*4 and its derivatives at 10^5 cfu/plant. Nodules were scored at the 21st day after infection from plants grown in growth pouches. Data are means +/− SEM of 20 plants each.

plished by the construction and phenotypic characterization of specific, genetically defined mutants that were impaired in their EPS but not in their LPS production. Although surface polysaccharides of *B. japonicum* have been studied in detail at the biochemical level, this work constitutes the first report of specific, genetically defined exopolysaccharide mutants of *B. japonicum*. It is known that LPS performs crucial functions in the establishment of the *Glycine*/*Bradyrhizobium* symbiosis, since LPS mutants of *B. japonicum* are unable to form effective nodules on soybean (Stacey *et al.* 1991). To obtain information about EPS function, it was essential to construct mutants with defects exclusively in EPS and not in LPS. In *B. japonicum*, EPS and LPS differ in their sugar composition in that only EPS contains galactose, whereas LPS does not. The *exoB* gene of *Rhizobium* species has been shown to be involved in the production of galactose-containing polysaccharides. Therefore, a mutation in this gene in *B. japonicum* should give rise to an EPS-specific mutant.

Using interspecies complementation, we isolated a 9.1-kb *Eco*RI fragment from *B. japonicum* having functional homology to the *R. meliloti exoB* gene. Defined mutants of *B. japonicum* within this fragment were constructed and their phenotypes were analyzed both biochemically and in symbiosis with the host plant *G. max*. In *R. meliloti* and *R. leguminosarum*, the *exoB* gene encodes UDP-glucose 4′-epimerase, an enzyme that interconverts UDP-glucose and UDP-galactose (Buendia *et al.* 1991; Canter Cremers *et al.* 1990). The *B. japonicum exoB* mutants also lacked UDP-glucose 4′-epimerase activity. Furthermore, DNA sequence analysis of the region containing the deletion revealed significant sequence homology with the deduced amino acid sequence of the ExoB gene product from *R. meliloti* (unpublished data of our laboratory). Based on these observations, we conclude that the *B. japonicum* deletion mutants lack the *exoB* gene.

Deletion of an *exoB*-like gene did not affect LPS biosynthesis in *B. japonicum*. This was as expected based on the following observations. In *R. meliloti* and *R. leguminosarum*, UDP-glucose 4′-epimerase is involved in the synthesis of galactose-containing polysaccharides, i.e., in EPS and LPS synthesis of these bacteria (Buendia *et al.* 1991; Canter Cremers 1990). In contrast, LPS of *B. japonicum* 110 does not contain galactose (Puvanesarajah *et al.* 1987), and therefore there is no requirement for UDP-glucose 4′-epimerase in biosynthesis of LPS.

Table 2. Competitiveness of *exoB* mutants of *Bradyrhizobium japonicum*[a]

Inoculum mixture (wt:mutant)	Nodule occupancy by strain (%)		
	P29	ΔP5	ΔP22
$10^5:10^5$	54	0	0
$10^4:10^5$	ND	7	7
$10^3:10^5$	ND	46	44
$0:10^5$	100	100	100

[a]Competitiveness of mutants as determined by nodule occupancy. Mutant strains were inoculated with approximately 10^5 cfu/plant in mixture with different titers of the parent strain *B. japonicum* 110*spc*4. Each figure represents at least 60 nodules from 20 plants.

ExoB mutants formed effective nodules on *G. max*. This indicates that the normal, wild-type, galactose-containing EPS structure of *B. japonicum* is not a prerequisite for the establishment of an effective symbiosis with *G. max*. However, nodules induced by *exoB* mutants appeared with a delay of about 5 days, suggesting that early stages of the symbiotic interaction were disturbed. Further evidence for this is the greatly reduced competitiveness of the deletion mutants relative to the wild type when applied at similar inoculum titers. This phenotype is most likely attributable to the altered EPS structure, since the LPS structure of *exoB* mutants is not impaired. Our results imply that the normal, wild-type, galactose-containing EPS structure of *B. japonicum* contributes to competitiveness for nodulation of soybean.

B. japonicum exoB mutants produce EPS that apparently lacks galactose. Therefore, the structure of this mutant EPS differs from that produced by the wild type which contains one galactose residue in a pentasaccharide repeating unit (Mort and Bauer 1982). In *R. meliloti*, a second EPS is synthesized in mutants that have an altered EPS I. This EPS II consists of a disaccharide repeating unit of β-(1-3)-linked acetylated glucose and pyruvylated galactose. It cannot, however, function in the place of EPS I in the infection process in alfalfa (Pühler *et al.* 1991). Stationary-phase cultures of *B. japonicum exoB* mutants produce only half of the EPS amounts synthesized by the wild type. It is not clear whether the altered EPS structure in *B. japonicum exoB* mutants is the direct consequence of the deletion in these strains, resulting in a modified biosynthesis pathway and/or a disturbed regulation of *exo* gene expression. Alternatively, there might exist a second EPS in *B. japonicum* which has not been detectable so far, since the structure reported by Mort and Bauer (1982) is the predominant form of EPS, whereas the synthesis of the second EPS would be down regulated in the wild type.

The detailed chemical analysis of the polysaccharides produced by the *B. japonicum* EPS mutants should provide valuable information about EPS structural features important for high competitiveness. Not only should EPS produced by free-living bacteria be analyzed but also EPS produced in the bacteroid state, because it is known that rhizobial exopolysaccharides undergo compositional changes induced either by root exudate (Bhagwat and Thomas 1984) or during differentiation into bacteroids (Streeter *et al.* 1992). Therefore, the EPS predominantly produced under free-living conditions might not represent the relevant structure during symbiotic interactions with the plant in different developmental stages.

A correlation between delayed nodulation and a reduced competitiveness similar to that found in the present study has also been described by Hahn and Hennecke (1988). Their analyses involved mutants carrying large deletions in symbiotically relevant DNA regions of *B. japonicum* 110*spc*4. These results imply a role for the speed of nodulation in competitiveness. In soybean, the number of root nodules formed is largely determined by the host plant and is the result of a complex regulatory process involving both root- and shoot-derived effector substances. The first nodule primordia formed in response to inoculation lead to an arrest in the development of further primordia (Caetano-Anollés and Gresshoff 1991). This phenomenon has been termed autoregulatory response (Pierce and Bauer 1983). It has been assumed that the onset of the autoregulatory response in soybean following infection with a superior strain might be involved in the exclusion of a slow-to-nodulate strain (reviewed by Triplett 1990).

A relationship between EPS production and competitiveness for nodulation of soybean has already been suggested in a number of studies. Tn*5*-induced mutants of *B. japonicum* 110 showing reduced competitiveness also exhibit pleiotropic defects in both EPS and LPS synthesis (Bhagwat *et al.* 1991). The authors hypothesized that the reduced competitive ability was due to the alterations in EPS and not in LPS. It has recently been discovered that some strains of *B. japonicum* produce large amounts of polysaccharide in the bacteroid state, giving rise to larger symbiosomes, a phenomenon apparently positively correlated with high competitiveness (Streeter *et al.* 1992). Tn*5*-induced mutants of *R. fredii* with reduced EPS production exhibited an increased competitiveness for nodulation of the primitive soybean cultivar 'Peking' (Zdor and Pueppke 1991). Although EPS is not required for effective nodule formation, the host plant is apparently infected by bacteria having specific EPS amounts and/or structures, since alterations in these traits lead to alterations in competitiveness.

The previously stated generalization, that EPS is not necessary for effective nodule formation in determinate nodule-type legumes (Diebold and Noel 1989; Hotter and Scott 1991), should be reevaluated based on the results of the present study. It is clear that mutation of the EPS does not result in a blocked infection process as observed in plants with indeterminate nodules, since effective nodules are still formed. Nevertheless, our data show that EPS of *B. japonicum* contributes significantly to optimal development of the symbiosis during the early stages. This implies a generalized importance of rhizobial EPS in the infection process both in indeterminate and determinate nodule-type symbioses.

MATERIALS AND METHODS

Growth of bacteria.

Rhizobial strains were grown at 28° C in 20E medium (Werner *et al.* 1975) containing yeast-extract, glycerol, mannitol, and salts buffered with 20 mM MES pH 6.5 (20E MES) or in a mineral medium (Tully 1985) with succinate (SMM), galactose (GMM), or xylose (XMM) as sole carbon source. Addition of Congo red (4 mg/L) to the agar media allowed an improved discrimination of the EPS mutants from the wild type using their staining behavior. Staining of *R. meliloti* EPS with the fluorescent dye Cellufluor white (cfw) (Polysciences, Frankfurt, Germany) was performed as previously described (Müller *et al.* 1988).

DNA analysis.

DNA manipulations and analyses were carried out as described (Sambrook *et al.* 1989) except that commercial nonradioactive detection kits based on digoxigenin or biotin labeled DNA probes were used in Southern hybridization experiments.

Construction of a *B. japonicum* cosmid gene bank.

Total DNA of *B. japonicum* 110*spc*4 was partially restricted with *Eco*RI and size fractionated by electrophoresis in a 0.5% (w/v) low melting agarose gel. Fragments 20–30 kb in size were extracted from the gel and ligated with cosmid vector pVK100 (Knauf and Nester 1982). After *in vitro* packaging, *E. coli* strain S17-1 (Simon *et al.* 1983) was transfected.

Genetic complementation of *R. meliloti* mutant H36.

E. coli S17-1 harboring the cosmid gene bank was directly used for conjugational transfer of the individual clones en masse to the *R. meliloti* mutant H36 (Buendia *et al.* 1991). Selection was carried out on TY plates containing streptomycin (500 mg/L) and neomycin (100 mg/L). Complemented transconjugants were identified by the addition of 0.02% Cellufluor white to the agar medium resulting in bright colonies under UV light (302 nm).

Homogenotization of *B. japonicum*.

Mutants were constructed by marker exchange as previously described (Hahn and Hennecke 1984). The 9.1-kb *Eco*RI fragment of *B. japonicum* DNA was cloned in pSUP102 (Simon *et al.* 1983), resulting in plasmid pBJ1. Derivates of pBJ1 carrying the *aph* gene of pUC4K (Vieira and Messing 1982) were transferred to *B. japonicum* 110*spc*4 (Hahn and Hennecke 1984) by mating with *E. coli* S17-1 for 3 days on PSY agar at 28° C. After mating, the cells were resuspended in 0.9% NaCl and appropriate quantities were plated on PSY agar containing kanamycin (100 mg/L) to select for integration events and chloramphenicol (50 mg/L) and spectinomycin (200 mg/L) to inhibit growth of *E. coli*. The resulting clones were counterselected against cointegration of pSUP102 by streaking on PSY agar containing tetracyclin (100 mg/L) and chloramphenicol (50 mg/L). All mutant strains were purified by two consecutive single-colony passages prior to further characterization.

Cell-free extracts and enzyme assay.

Cells were grown in 400 ml of 20E-MES to an OD_{600} of approximately 0.5, washed twice in 0.9% NaCl, and the pellets were stored frozen until used. The cells were resuspended in 3 ml of 25 mM potassium phospate buffer (pH 6.8) containing 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and broken by two passages through a French pressure cell. Intact cells and debris were removed by centrifugation for 10 min at 15,000 × *g*. The supernatant was centrifuged at 150,000 × *g* for 60 min. The clear supernatant was used for determination of UDP-glucose 4′-epimerase according to Postma (1977), except that UDP-galactose-dehydrogenase (Boehringer, Mannheim, Germany) was adjusted to 60 mU/ml, UDP-galactose to 0.6 mM, and NAD to a concentration of 2 mM.

LPS and EPS analysis.

Bacterial protein was determined as described by Herbert *et al.* (1971). LPS of Proteinase K digested cells was analyzed by Na-desoxycholate (DOC)-PAGE as described by Krauss *et al.* (1988) except that DOC concentration in the gels was reduced to 0.25%. Cells were suspended in distilled water, an equal volume of Laemmli sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.001% bromphenol blue) was added and the suspension was heated for 10 min at 100° C. Proteinase K (0.29 mg/ml) was added, and protein was digested 1.5 h at 37° C. The samples were then adjusted to 0.25% DOC and heated for 5 min at 100° C. Samples equivalent to 10 μg of bacterial protein were loaded per lane. The gels were silver-stained according to Hitchcock and Brown (1983) except that gels were fixed with 40% ethanol in 5% acetic acid for 2 days with two subsequent washes for 10 min with 25% isopropanol in 7% acetic acid prior to oxidation with periodic acid. LPS from *Salmonella minnesota* (Sigma, Deisenhofen, FRG) was used as standard.

To isolate EPS, 10-ml cultures of *B. japonicum* grown in SMM to late logarithmic phase were adjusted to 20% ethanol and stored at 4°C for 7 days to solubilize the CPS coat. Therefore the EPS analyzed includes both soluble and capsular extracellular polysaccharide fractions. The supernatant was freeze-dried, redissolved in 1 ml of H_2O, and EPS was precipitated with 90% ethanol. The pellet was washed with ethanol, dried, and redissolved in H_2O for further analysis. Total EPS was determined using the anthrone reagent (Herbert *et al.* 1971) with glucose as standard. EPS was hydrolyzed with 1 M HCl at 100° C for 4 hr. Galactose content of this hydrolysate was analyzed enzymatically (Kurz and Wallenfels 1970) using a commercial preparation of cloned galactose dehydrogenase of high specific acivity (Sigma, Deisenhofen, Germany). The TMS derivatives of sugars in the hydrolysate were produced by incubation for 50 min at 80° C in 99% bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (Sigma, Deisenhofen, Germany). The products were analyzed by capillary GC-MS using a 30 m DB-1 column (I&W Scientific) and a temperature program starting with 1 min at 140° C and a subsequent linear increase of 2° C/min up to 220° C.

Growth of plants and competition assay.

Seeds (*Glycine max* 'Preston') were surface sterilized by immersion in 30% (v/v) H_2O_2 for 10 min, washed 10 times with water, soaked for 6 h, and washed again. The seeds were then placed on nitrogen-free nutrient agar (Werner *et al.* 1975) and grown for 2 days in a growth chamber at 25° C (16 hr light/8 hr dark). Plants were then transferred to growth pouches or Leonard jars. In growth pouches, plants were grown for an additional day prior to infection. When used for plant infection, *B. japonicum* strains were grown in SMM. For competition experiments, *B. japonicum* 110*spc*4 was applied as a mixed inoculum with its derivatives in a 1:1, 1:10, and 1:100 ratio. Mutant strains were always inoculated at 10^5 cfu per plant. Nodule occupancy was tested by antibiotic resistance and colony morphology of the reisolated bacteria. Nodules surface were sterilized by immersion in 96% ethanol for 2 sec. Excess ethanol was burnt, and the nodules were individually crushed in 20Q medium (same as 20E except that mannitol concentration was increased to 36.44 g/L). The suspension was subsequently streaked in parallel on 20Q agar and 20Q agar supplemented with kanamycin (100 mg/L) to differentiate the mutants from their parent strain *B. japonicum* 110*spc*4. Cycloheximide and spectinomycin (100 mg/L each) were included in the agar media to prevent growth of nodule surface derived contaminants.

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