The Regulation of Exopolysaccharide Production Is Important at Two Levels of Nodule Development in *Rhizobium meliloti*

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We show that two exopolysaccharide overproducing Tn*5* mutants of *Rhizobium meliloti*, *exoR* and *exoS*, have distinct symbiotic defects. While the *exoR* mutant is unable to colonize nodules, the *exoS* mutant retains that ability but varies in its ability to produce nitrogen-fixing nodules. We correlate these defects with different degrees of exopolysaccharide overproduction and growth impairment. We further show that the *exoR* mutant is able to enter developing infection threads but is unable to invade nodule cells. The *exoR* mutant gives rise to spontaneous pseudorevertants containing second-site suppressor mutations that decrease exopolysaccharide synthesis. These pseudorevertants form nitrogen-fixing nodules. Although the suppressor mutations have the opposite effect on exopolysaccharide production compared to the *exoS*::Tn*5* mutation, they consistently map to the *exoS*::Tn*5* region and belong to the same genetic complementation group as defined by transposon insertion mutations. The effect of the suppressor mutations on exopolysaccharide production is correlated with effects on the expression of *exo* genes involved in exopolysaccharide synthesis. Finally, we provide evidence that the *exoR* gene is not required for the regulation of exopolysaccharide synthesis by ammonia.

Additional keywords: infection thread, instability, mucoid, succinoglycan.

In recent years, a number of groups have shown that nodule invasion or development induced by rhizobial symbionts of several different plant hosts such as alfalfa, pea, clover, and *Leucaena* requires bacterial exopolysaccharide (EPS) (reviewed by Leigh and Coplin 1992; Gray and Rolfe 1990). In particular, nodule invasion of alfalfa by *Rhizobium meliloti* requires succinoglycan, an acidic heteropolysaccharide comprised of repeating octasaccharide subunits each containing seven β-linked glucoses and one β-linked galactose as well as one acetyl, one pyruvyl, and one succinyl modification (Aman et *al.* 1981). The polysaccharide is secreted in both high and low molecular weight forms (Leigh and Lee 1988) that vary in their numbers of repeat units (Battisti *et al.* 1992). Tn*5*-induced mutants that lack or produce altered forms of succinoglycan were first identified by their appearance on LB agar plates containing the flourescent dye Calcoflour and induce nodules that form abortive infection threads and lack intracellular bacteria (Finan *et al.* 1985; Leigh *et al.* 1985; Leigh *et al.* 1987). Extensive genetic analysis has established that genes (*exo*) required for the synthesis of this molecule are clustered within a 24-kb region located on a 1,500-kb megaplasmid termed pSymB (Finan *et al.* 1986). Recently, the entire cluster of 19 *exo* genes was sequenced, and the roles of most of these genes in the biosynthesis, modification, and export of succinoglycan were established (see Leigh and Walker 1994).

In a previous study, two unlinked recessive chromosomal mutations, *exoR95*::Tn*5* and *exoS96*::Tn5 were described (Doherty *et al.* 1988). These mutations resulted in a mucoid colony phenotype caused by overproduction of EPS, indicating that the ExoR and ExoS gene products functioned as negative regulators of EPS synthesis. While the *exoS* mutant appeared to remain responsive to inhibition of EPS synthesis by ammonia, the *exoR* mutant was reported to have lost this responsiveness (Doherty *et al.* 1988). The *exoR* gene was subsequently cloned and sequenced. While it appeared to regulate transcription, it had no homology to any known bacterial transcriptional regulators (Reed *et al.* 1991). The sequence of the *exoS* gene has not yet been reported. The *exoR* and *exoS* mutants were reported to have nodulation defects, the former more marked than the latter (Doherty *et al.* 1988). *exoR* induced two kinds of nodules: white nodules that failed to fix nitrogen (Fix^-) and lacked recoverable bacteria, and pink nodules that fixed nitrogen (Fix^+) and contained bacteria. Notably, the bacteria recovered from the pink nodules were exclusively nonmucoid, having lost the EPS overproducing phenotype of the original mutant. These arose as a consequence of unlinked suppressors that appeared to restore EPS to normal levels; the original *exoR* mutation remained unaltered. In contrast, the nodulation defect of the *exoS* mutant was milder. Although some variability in the Fix phenotype was reported, the nodules were predominantly Fix^+ and contained bacteria that retained the mucoid phenotype.

In this report, we further characterize the nodulation phenotypes of the *exoR* and *exoS* mutants as well as the *exoR* pseudorevertant. We also describe EPS synthesis more thoroughly, show the growth defects that these mutants suffer, and correlate these data with the nodulation defects. Finally, we show that the *exoR* suppressor mutation maps to the *exoS* locus as do additional, independently isolated suppressors.

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RESULTS

The *exoR*::Tn*5* and *exoS*::Tn*5* symbiotic defects.

Previous studies with these two mutants (summarized above), suggested the possibility that only nonmucoid derivatives of the *exoR* mutant were able to invade nodules. By corollary, an *exoR* culture lacking such derivatives should induce empty nodules. Since we had observed that nonmucoid derivatives appear with time in *exoR* colonies, we tested this hypothesis by inoculating alfalfa with cultures grown from young (4 day) and old (28 day) colonies.

Table 1. Nodule occupancy of *exoR*::Tn5 and *exoS*::Tn5[a]

Strain	Average cfu/nodule	Colony appearance	Plant growth
Rm 1021	1.22×10^5	Nonmucoid	Fix^+
exoR::Tn*5* (four day)	6.6×10^2	Mucoid	Fix^-
exoR::Tn*5* (28 day)	1.75×10^5	Nonmucoid[b]	Fix^+
exoS::Tn*5*	5.16×10^4[c]	Mucoid	Fix var.[d]

[a] Four plants were inoculated each with Rm 1021 and *exoR*::Tn*5* samples as described. Six plants were inoculated with *exoS*::Tn*5*. Numbers of nodules examined for nodule occupancy: Rm 1021, 10; 4-day *exoR*, 34; 28 day *exoR*, 23 and *exoS*, 21. Nodule occupancy shown for 28- day *exoR* reflects only those nodules that contained recoverable bacteria.

[b] Six out of 15 nodules contained low numbers of mucoid *exoR*::Tn*5* as well as the predominant non-mucoid derivatives.

[c] Average for pink and white nodules.

[d] Two out of the six plants were Fix^+.

Nodules induced by cultures from 4-day colonies were exclusively white and bluntly elongate. Only low numbers of bacteria could be recovered and these gave rise exclusively to colonies that exhibited the mucoid appearance of the original *exoR* mutant (Table 1). All infected plants were stunted and chlorotic, indicating a lack of nitrogen fixation as established previously for *exoR* inoculated plants containing only white nodules (Doherty *et al.* 1988). Although nodules were white and Fix^-, they were larger and more elongate than those formed by *exo* mutants lacking or producing altered forms of EPS. Light microscopy of several representative 4-day *exoR* nodules confirmed the presence of a meristem (Fig. 1B). However, in contrast to the wild-type nodule (Fig. 1A), the nitrogen-fixing zone appeared to have no bacteria. Instead, the region contained large numbers of amyloplasts (Fig. 1C). We were able to visualize the presence of infection threads which contained bacteria that appeared to undergo changes in shape consistent with bacterial disintegration (Fig. 1D). No

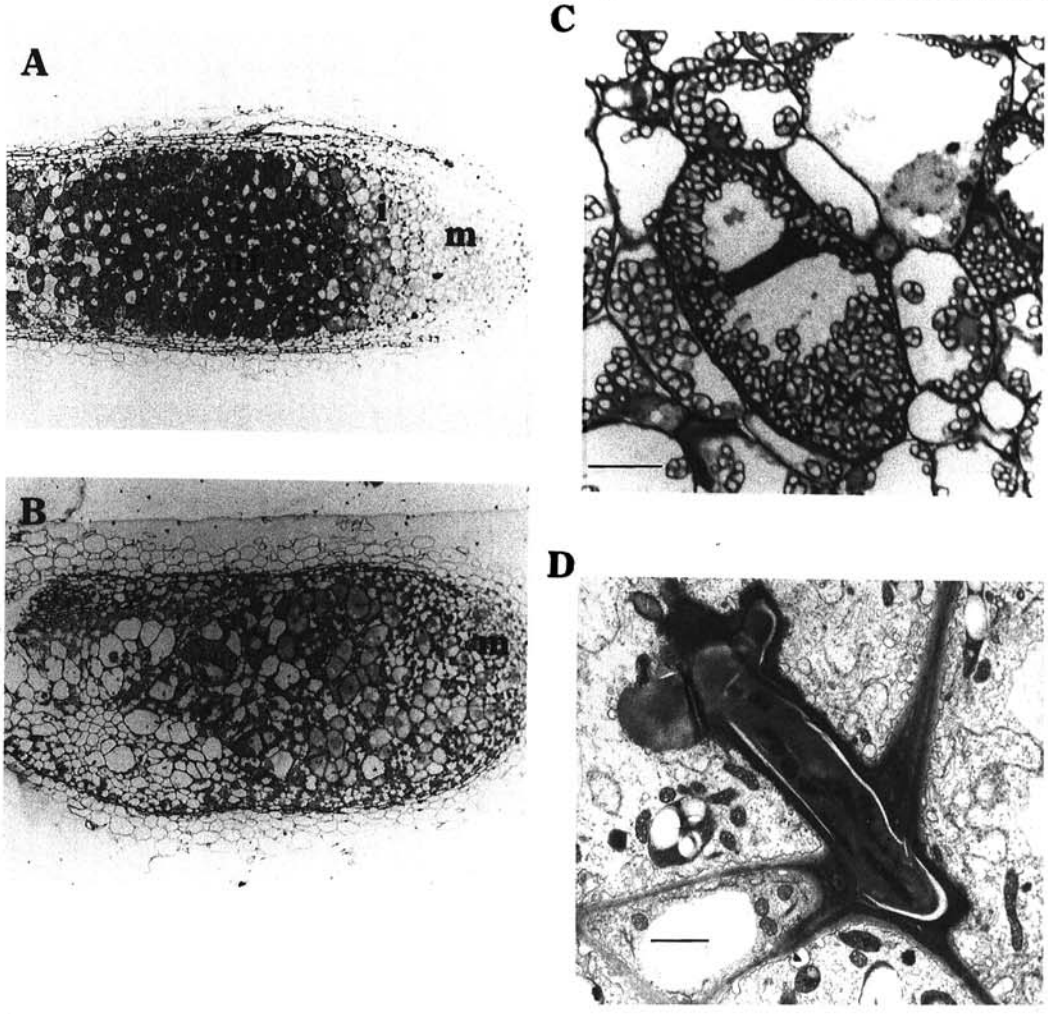

Fig. 1. Light micrographs of nodule thick sections. **A,** Wild-type Rm 1021; **B,** 4-day-old exoR::Tn*5* cultures. Longitudinal thick sections were obtained from 4-wk-old nodules. Total magnification was 80×. The meristematic zone (m), infection zone (i), and nitrogen-fixing zones (nf) are indicated. **C,** Light micrograph of amyloplasts. Bar equals 10 μm. D, transmission electron microscopy of infection thread. Bar equal 1 μm.

intracellular bacteria were observed; thus, the low numbers of bacteria that are recovered from *exoR* nodules seem to originate from infection threads. These results show that the *exoR* mutant has an invasion deficiency that occurs after infection thread penetration.

Inoculation with cultures grown from 28-day cultures produced a contrasting situation. Fifteen out of 23 nodules were pink and elongate and contained bacteria that were predominantly nonmucoid; these were recovered in numbers similar to those obtained from nodules formed by the wild type strain Rm 1021 (Table 1). The remaining eight nodules were white and either bluntly elongate or round with no recoverable bacteria. Plant growth resembled that of wild-type infected plants. These results indicate that, although the *exoR* mutant is unable to invade nodules, if a subpopulation of nonmucoid derivatives is present, these will invade and fix nitrogen. This conclusion was confirmed by studies in which plants were inoculated with a cell suspension that contained a mixture of *exoR* and a nonmucoid derivative in a ratio of 50 to one. The vast majority of nodules contained only the nonmucoid derivative.

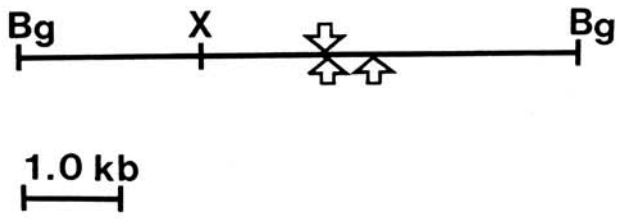

Fig. 2. Map of *exoS* region. Restriction sites defining the 5.3-kb *Bgl*II fragment and the 3.7-kb *Xba*I-*Bgl*II fragment are shown. Arrows represent the locations of Tn*5* insertions; on top, the original genomic *exoS96*::Tn insertion, and on the bottom the two insertions that eliminated complementation.

We also examined nodules formed by the *exoS* mutant using 4-day-old colonies. Out of 178 nodules visually examined, the majority were white and either round or elongate. Only 30 were either pink and elongate; four were pink and round. We examined seven pink (round or elongate) and 15 white (round or elongate) nodules for nodule occupancy and found averages of 4.0×10^4 and 5.6×10^4 bacteria per nodule, respectively. In every case, all bacteria retained the mucoid phenotype. Two out of the six plants were Fix$^+$ with growth similar to plants inoculated with the wild-type strain. The remaining four plants were Fix$^-$ in spite of the presence of pink nodules and high nodule occupancies (Table 1). Thus, in contrast to *exoR*, the *exoS* mutant invaded nodules; however, nitrogen fixation was diminished relative to the wild-type strain.

Mapping and complementation analysis of the suppressor mutation of *exoR*::Tn*5*.

One nonmucoid derivative of *exoR*::Tn*5* obtained from a nodule was used to determine the location of the spontaneous mutation leading to the nonmucoid phenotype. Rm 7300, which bears a Tn*5*-233 transposon marker closely linked to the wild-type suppressor locus (see Materials and Methods) was transduced into Tn*5* insertion mutants in *exoA,B,C,D,F,* and *H*. All of the gentamicin/spectinomycin resistant transductants were resistant to neomycin, indicating no transductional linkage of the Tn*5*-233 marker to any of these *exo* loci. When Rm 7300 was transduced into Rm 7096 (*exoS*::Tn*5*), however, 78% of the transductants were neomycin sensitive indicating linkage to the *exoS*::Tn*5* locus; therefore, this suppressor is termed *exoS**.

To determine the number of nodules that were occupied by strains containing suppressor mutations in the same locus, nine nodules from three plants inoculated with a culture grown from a 28-day *exoR* colony were crushed and plated on

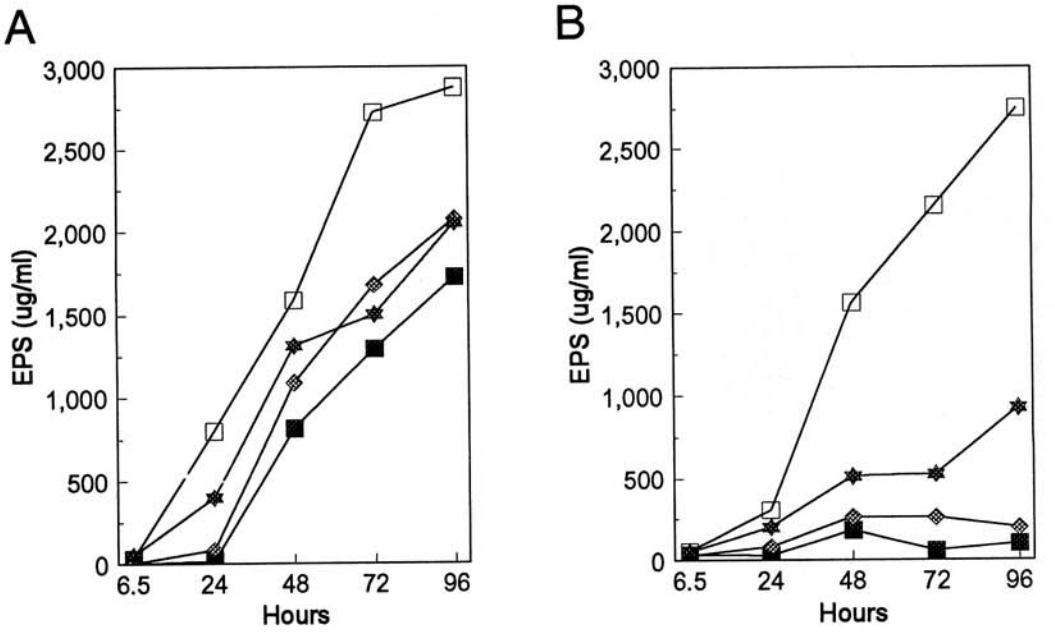

Fig. 3. EPS production in the absence **(A)** and presence of **(B)** of NH_4Cl. Points represent averages of duplicate meaurements. Rm 1021, closed box; *exoR*::Tn*5*-233*exoS**, diamond; *exoS*::Tn*5*, star; *exoR*::Tn*5*, open square.

LB agar. Two nonmucoid colonies arising from each nodule were then grown and analyzed for linkage of the nonmucoid phenotype to the Tn*5*-233 tag in Rm 7300. Eight out of nine pairs of nonmucoid derivatives each exhibited linkages ranging between 69 and 76%, indicating that the vast majority of the nodules examined contained suppressors that were genetically similar to *exoS**. Two nonmucoid derivatives obtained from the remaining nodule were unlinked to the tag and were not further characterized.

Nonmucoid derivatives of *exoR*::Tn*5* were also obtained directly from colonies. Several 28-day-old *exoR* colonies were restreaked on LB agar and screened for a mucoid or nonmucoid appearance. As many as 50% of the observed colonies were nonmucoid. Several nonmucoid derivatives arising from each original colony were then tested for linkage of the suppressor mutation to the tag. Out of a total of 17 colonies analyzed, 15 were linked with tranductional frequencies ranging from 74 to 79%, while the remaining two were unlinked.

We further examined the relationship between *exoS*::Tn*5* and *exoS** by complementation analysis. Doherty *et al.* (1988) had identified cosmid clone pM13 that contained the *exoS* locus and complemented the mucoid phenotype of *exoS*::Tn*5*. We isolated an identical cosmid and found that it also complemented the nonmucoid phenotype of *exoR*::Tn*5exoS**. Random Tn*5* insertion mutagenesis of pM13 yielded two insertions that eliminated both complementation functions. Restriction mapping showed that these insertions were closely linked to the point of the original *exoS*::Tn*5* mutation. Subcloning identified a 3.7-kb *Xba*I-*Bgl*II fragment containing the *exoS* locus that complemented both kinds of mutations (Fig. 2). Therefore, both *exoS*::Tn*5* and *exoS** are contained within a 3.7-kb region and are disrupted by the same transposon insertions.

Analysis of EPS production, growth, and *exo* gene expression.

Since the results reported above suggested a correlation between EPS overproduction and the failure to nodulate successfully, we studied EPS production in pertinent strains. Since EPS synthesis is known to vary with culture age and growth conditions including nitrogen source (reviewed by Sutherland 1979), we monitored EPS over time in the presence and absence of ammonia in M9 medium. The *exoR*::Tn*5* mutant produced the highest levels of EPS, followed in order of diminishing levels by *exoS*::Tn*5*, *exoR*::Tn*5*-233*exoS** and wild-type strains (Fig. 3). The differences between *exoR*::Tn*5* and the other strains were particularly marked in the presence of ammonia at later time points, but were also clear at all times under both conditions. Notably, in both the presence and absence of ammonia, higher EPS production appeared to occur at the expense of growth (Fig. 4). Ammonia had a clear inhibitory effect on EPS production as expected (Fig. 3). This effect of ammonia appeared to decrease in strains that produced more EPS. However, the effect was still noticeable in the highest producer, *exoR*::Tn*5*, at 24 hr.

The effects of the *exoR*::Tn*5* mutation and the *exoS** mutation on EPS production were paralleled by their effects on the expression of genes involved in EPS synthesis. β-Galactosidase activity arising from an *exoY-lacZ* fusion was measured in the presence and absence of ammonia (Fig. 5). Gene expression was always much higher in the *exoR*::Tn*5* strain than in *exoR*::Tn*5exoS** or wild-type strains. Similar results were observed for alkaline phosphatase activity arising from an *exoP-phoA* fusion (not shown).

Wild-type *R. meliloti* strains produce EPS in two molecular weight ranges, and the low molecular weight fraction can partially rescue the nodule invasion defect of *exo* mutants that fail to produce EPS (Battisti *et al.* 1992). Accordingly, we

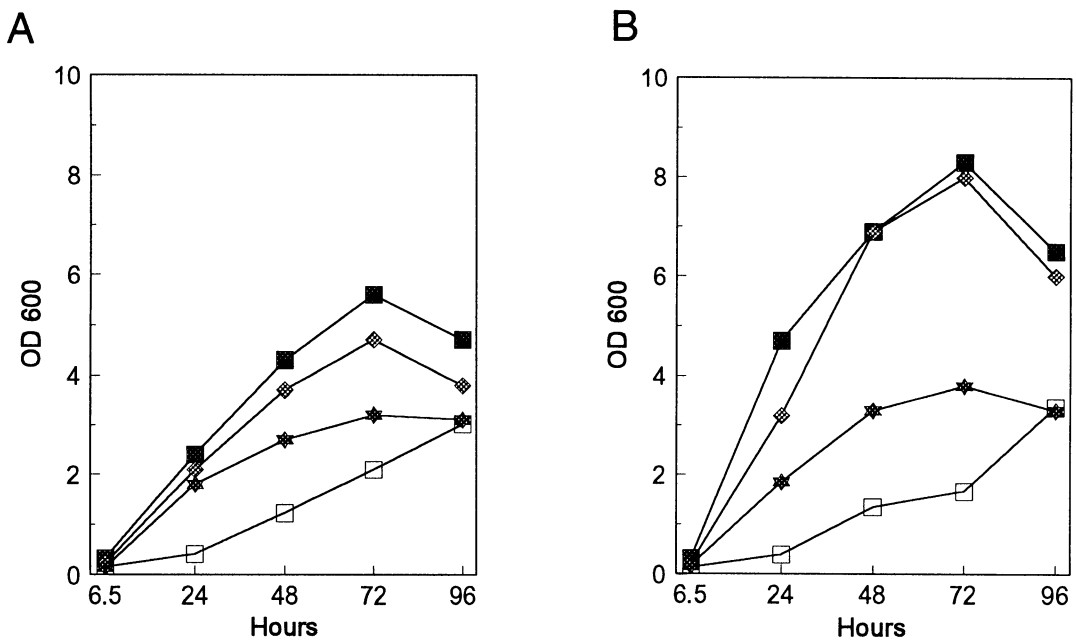

Fig. 4. Growth of cultures in the absence **(A)** and in the presence **(B)** of NH_4Cl. Points represent average of duplicate measurements. Rm 1021, closed square; *exoR*::Tn*5*-233*exoS**, diamond; *exoS*::Tn*5*, star; *exoR*::Tn*5*, open square.

wondered if the nodulation defects of the *exoR* and *exoS* strains could be related to a change in the molecular weight distributions of EPS. Supernatants from cultures of wild type, *exoR*::Tn*5exoS**, *exoS*::Tn*5*, and *exoR*::Tn*5*, grown for 3 days in low salt medium, were fractionated into high and low molecular weight forms and measured. Although the proportion of high molecular weight EPS increased with increasing total production, all strains still produced at least half low molecular weight EPS (data not shown). We also checked for detectable differences in EPS structure by NMR spectroscopy, and found none.

DISCUSSION

Previous studies have shown that exopolysaccharide synthesis by *R. meliloti* is necessary for the invasion of developing nodules (Leigh *et al.* 1985). In the present study we turn to the question of exopolysaccharide overproduction. Our results suggest that negative regulation of exopolysaccharide synthesis must occur at two stages of nodule development. First, as shown with the most extreme overproducer, *exoR*, some degree of regulation is necessary for nodule invasion to occur. The lack of EPS regulation by a 4-day-old *exoR* strain results in infection thread penetration but nodule cell invasion remains impaired. The correlation between overproduction and the failure to invade is strengthened by the observation that both invasion and near-normal levels of EPS are restored in the same suppressor strains. Of interest is the observation that the general elongate morphology of nodules induced by a 4-day-old *exoR* strain resembles that of the wild type. It would thus seem possible that infection thread penetration in the absence of nodule cell invasion permits further stages of nodule development. Secondly, as demonstrated by the more moderate overproducer *exoS*, some constraints on EPS biosynthesis must also exist within the nodule for the bacteroids to fix nitrogen optimally even if invasion does occur. Regulation of EPS is therefore essential not only for nodule cell invasion, but also for the development of the symbiotic process after nodule cell invasion. The symbiotic phenotype of the *exoS* strain is consistent with the observation that Tn*phoA* fusions to *exo* genes involved in succinoglycan synthesis show alkaline phosphatase activity only within the invasion zone of the nodule, not in the bacteroid development zone (Reuber *et al.* 1991). Thus, EPS production, which may not normally occur in the bacteroid zone, may actually be deleterious if it does occur.

It is interesting to note that in the case of another class of EPS overproducers, *exoX* (Zhan and Leigh 1990; Reed *et al.* 1991), nitrogen-fixing nodules are consistently produced. This contrasting situation may be due to differences in the modes of regulation exercised by the regulatory genes. While *exoR* and *exoS* affect gene expression, *exoX* appears to act posttranslationally. The effects that these genes have on EPS synthesis during nodulation may differ.

An additional conclusion regarding EPS overproduction and invasion emerges from the fact that when plants are inoculated with a mixture of normal producers and *exoR* overproducers, the normal producers dominate and invade successfully. Apparently, the deleterious effect of EPS overproduction on nodule cell invasion is restricted to the overproducing cells and does not affect normal producers in the population.

EPS overproduction could affect nodule invasion and the development of nitrogen fixation in several possible ways. First, the defects could arise directly from the physical properties of the EPS such as viscosity, acidity, or hydrophilicity, interfering somehow with normal nodulation. For example, an excess of viscous polymer could physically retard the movement of the bacteria within the infection thread. Second, excess EPS could mask some required molecular determinant on the surface of the plant or bacterium, thereby interfering with some molecular recognition event. It is notable that in the first two cases the effect of excess EPS on invasion would have to be localized to the overproducing cells and could not

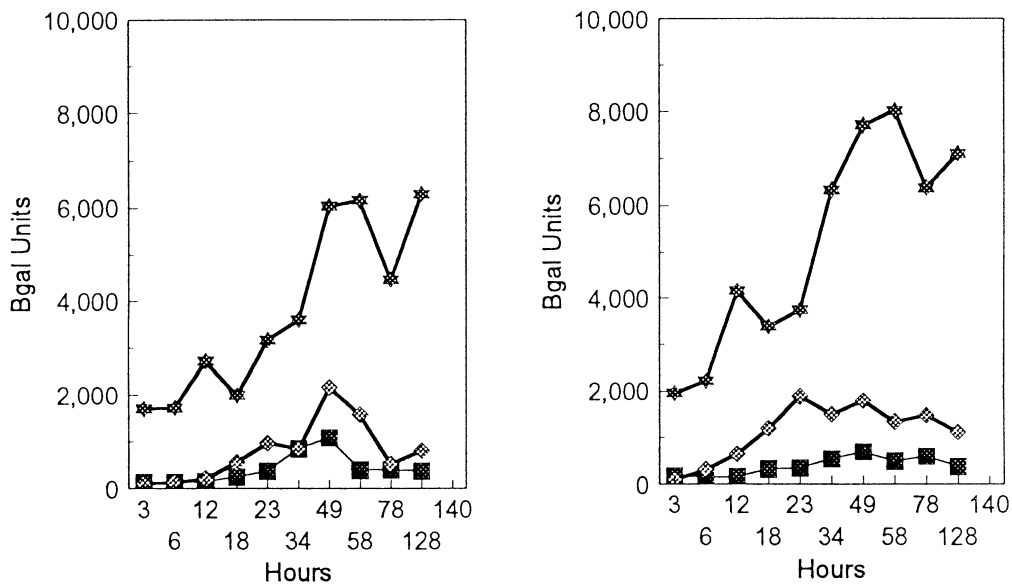

Fig. 5. β-Galactosidase activity of *exoY-lacZ* fusion in the presence (left) and absence (right) of NH_4Cl. Rm 1021, closed square; *exoR*::Tn*5exoS**, diamond; *exoR*::Tn*5*, star.

extend to normal producers when they are present. Third, EPS overproduction could be accompanied by a change in the chemical composition of the EPS, causing it to lose its normal symbiotic function. However, we did not detect any such change beyond a qualitative shift in the proportions of high and low molecular weight succinoglycan. Fourth, EPS overproduction could be accompanied by a change in some other physiological property of the bacterium, such as growth. Indeed, we noted a consistent inverse correlation between the level of EPS produced and the growth rate, as if EPS overproduction drains the cell of the physiogical resources needed for growth. Poor growth of the *exoR* mutant within the infection thread could impair the invasion process. In the case of the *exoS* mutant, such a physiological drain could also hamper carbon or nitrogen metabolism within the bacteroid, resulting in abnormal development or decreased nitrogen fixation.

In the course of our measurements of EPS synthesis, we observed decreased EPS synthesis in the presence of ammonia, as is commonly observed in bacteria (reviewed by Sutherland 1979). The effect of ammonia persisted in the *exoR*::Tn*5exoS** mutant, and even to some extent in the *exoR*::Tn*5* mutant, making it unlikely that the *exoR* gene product is required for sensing ammonia as previously suggested (Doherty *et al.* 1988; Reed *et al.* 1991).

We have observed that suppressor mutations which reduce the level of EPS synthesis arise in cultures at a frequency that increases with the age of the culture, and this loss of EPS production may be a general phenomenon among bacteria (Govan 1975). Amemura *et al.* (1977) described mutants of *Alcaligenes* var. *myxogenes* 10C3 that synthesized decreased levels of succinoglycan and observed that, whereas growing cultures produced these mutants at a continuous frequency of 5.0×10^{-7}, older cultures produced them at a frequency that increased over time to approximately 9.0×10^{-3}. It thus appears that succinoglycan synthesis can be modulated by mutational events that arise as a consequence of growth and/or cell division deficiencies. By comparison, *Pseudomonas aeruginosa* tends to lose its EPS-producing capacity when grown in the laboratory (Mian *et al.* 1978), and regains it in the course of human infection (Dogget *et al.* 1969).

The consistent localization to the *exoS* region of suppressor mutations that restore normal levels of EPS is striking. Reed *et al.* (1991) described two Tn*5* mutations within the *exo* gene cluster (*exoG*302 and *exoX(J)*319) that restore EPS synthesis to normal levels in *exoR* and *exoS* backgrounds. Thus, the potential for other spontaneous mutations that diminish EPS production would seem to exist, but were not observed. Notably, the *exoS* locus, to which the mutations belong, was previously identified as a regulatory locus (Doherty *et al.* 1988). The *exoS** mutations belong to the same genetic complementation group as the previously described *exoS*::Tn*5* mutation, but have the opposite effect on EPS production and *exo* gene expression.

Other bacteria can also regulate EPS production through genetic changes at specific loci. *Citrobacter freundii* and *Pseudomonas atlantica* regulate EPS synthesis by precise reversible excision and insertion events that are mediated by discrete insertion elements (Ou *et al.* 1988; Bartlett and Silverman 1989). *Haemophilus influenzae* type b capsular polysaccharide biosynthesis is mediated by a complex capsulation locus that contains directly repeated DNA flanking a bridge locus; spontaneous deletion of the polysaccharide export gene, *bexA*, from this locus results in capsule deficient strains (Kroll *et al.* 1988). In the case of our *exoS** mutations, we have not detected any physical rearrangements by Southern blotting with the cloned *exoS* region. It will be interesting to further characterize this locus and learn more about the regulatory function of the *exoS* gene.

MATERIALS AND METHODS

Strains and plasmids.

Bacterial strains, plasmids, and transducing phage are listed in Table 2.

Media.

Rhizobium meliloti was routinely grown in LB broth (Miller 1972) medium supplemented with 2.5 mM each $MgSO_4$ and $CaCl_2$ or on LB agar plates. LB agar was supplemented with Calcoflour (Cellufluor, Polysciences, Warrington, PA), autoclaved as a stock solution of 2% then added to a final concentration of 0.02% as needed to observe the presence of succinoglycan. For EPS analysis, either modified M9 or low salt media were used (Leigh and Lee 1988). In M9 medium, 5 mM sodium glutamate was substituted for ammonia, and mannitol (55 mM) was the carbon source. Biotin was added to 1 mM; final pH was 7.4. To measure EPS production in the presence of ammonia, medium was supplemented with NH_4Cl to 100 mM. Antibiotics were used at concentrations of (μg/ml): neomycin sulfate, 200; kanamycin sulfate, 25; gentamicin sulfate, 15; spectinomycin hydrochloride, 100 and chloramphenicol, 20.

EPS analysis.

For quantitation of EPS production, modified M9 medium was inoculated to an initial OD_{600nm} of 0.05 with cells obtained from overnight cultures grown in liquid LB medium. Duplicate 4 ml of M9 cultures were grown in 15 × 150 mm pyrex tubes with vigorous shaking at 30° C. EPS was assayed in supernatant solutions by the anthrone method (Loewus 1952). Size analysis of EPS was performed on supernatants of cultures grown for 3 days in low salt medium using Biogel A5 chromatography as previously described (Leigh and Lee 1988). Proton NMR spectroscopy was performed as before (Leigh and Lee 1988).

Genetic manipulations, cloning, and gene expression.

To map the nonmucoid suppressor of *exoR*::Tn*5*, Rm 1021 (wild type, steptomycin resistant) was randomly mutagenized with the gentamicin/spectinomycin resistance encoding transposon Tn*5*-233 (DeVos *et al.* 1986). The resulting streptomycin, gentamicin/spectinomycin resistant colonies were pooled, grown in LB broth, and the Tn*5*-233 insertions transduced as described (Finan *et al.* 1984) into the neomycin resistant *exoR*::Tn*5* suppressor strain selecting for neomycin, gentamicin/spectinomycin resistance. Several mucoid transductants were individually picked and the Tn*5*-233 marker transduced back into the *exoR*::Tn*5* suppressor strain. Linkage of the Tn*5*-233 marker to the suppressor mutation was scored by dividing the number of mucoid transductants by the

total number of transductants. A mucoid derivative containing a Tn*5*-233 insertion with 90% linkage to the locus of the suppressor mutation was chosen and transduced into Rm 1021 to obtain the tag in an otherwise wild-type background, resulting in strain Rm 7300. Additional suppressor mutations were mapped by transduction as described (Finan *et al.* 1984).

Gentamicin/spectinomycin resistant forms of Rm7095 and Rm7307 were constructed by exchanging Tn*5*-233 for Tn*5* as described (DeVos *et al.* 1986). Marker exchange was verified by neomycin sensitivity and by transduction of the transposon into Rm 1021 to generate the original *exoR* mutant phenotype. Tn*5* mutagenesis of cosmid pM13 was performed by introducing pM13 into *E. coli* MT614 using pRK600 as a helper plasmid, then from there into *E. coli* C2110 selecting for transfer of kanamycin resistance. *exoS* was subcloned from cosmid pM13 by ligating a 5.3-kb *Bgl*II fragment into *Bam*H-cut pSP329. The 3.7-kb fragment was then cloned by cutting at *Xba*I sites in the 5.3-kb fragment and in the vector multiple cloning site and ligating into *Xba*I-cut pSP329. The locations of genomic insertions relative to plasmid cloned DNA was determined by Southern hybridization.

For gene expression experiments, Lac$^-$ backgrounds were first constructed by transducing the Tn*5* insertion of strain KS1 into Rm7307 and Rm7312 to give Rm7309 and Rm7308, respectively. pD56*exoY*::Tn*3*HoKm was then introduced into these strains as well as into Rm7404. β-Galactosidase activity was measured according to Miller (1972).

Plant nodulation tests.

Alfalfa seeds, *Medicago sativa* 'Iroquois,' were surface sterilized for 20 min in a one-to-one solution of sterile distilled water and 5.25% sodium hypochlorite solution followed by three rinses of sterile distilled water of at least 15 min each. Seeds were germinated in the dark for 3 days on plates containing Jensen's agar (Leigh *et al.* 1985) then transferred to notched Jensen's agar plates through which developing seedlings emerged. Seedlings were incubated overnight at 25° C in a plant growth chamber (Percival, IA) with a light setting of 17 hr per day. Plants were inoculated with 10^7 cells suspended in 1 ml of sterile distilled water. Plants were incubated for 4 wk then analyzed by scoring for appearance, growth, and nodule development. To determine nodule occupancy, individual nodules removed from each plant were surface sterilized by immersion in a five-to-one mixture of sterile distilled water and 5.25% sodium hypochlorite solution for 2 min, followed by immersion in sterile water and liquid LB media for 1 min each. Nodules were crushed in 0.05 ml of LB media containing 0.3 M glucose for osmotic stabilization. Diluted or undiluted homogenates were then plated onto LB agar plates with appropriate antibiotic selection and incubated at 30° C for 3–4 days.

Fixation of nodules for light microscopy.

Nodules were fixed in 3.5% glutaraldehyde/1% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4, for 17 hr at room temperature then rinsed twice or more in cacodylate buffer. Nodules were postfixed in 1% aqueous OsO_4 in cacodylate buffer at 4° C for 3 hr then rinsed as above and dehydrated through a graded series of 35, 50, 70, 80, and 95% ethanol for 20 min each. This was followed by three changes in 100% ethanol of 15 min each, then two changes in acetone for 20 min. Samples were then placed in 25% Spurr's embedding medium for 5 hr, followed by 50% for 11 hr and 100% for 6 hr. Nodules were then embedded in fresh Spurr's medium and polymerized at 68° C.

Table 2. Bacterial strains, plasmids, and phage used in this study

Designation	Relevant characteristics	Source or reference
Rhizobium meliloti		
Rm1021	SU 47 Sm^r	F. Ausubel
Rm7095	Rm 1021*exoR95*::Tn*5*	Doherty *et al.* 1988
Rm7096	Rm 1021*exoS96*::Tn*5*	Doherty *et al.* 1988
Rm7300	Rm 1021ΩTn*5*-233 tag to *exoS* locus	This work
Rm7307	Rm 1021*exoR95*::Tn*5*-233*exoS**	This work
Rm7308	Rm1021*exoR95*::Tn*5*-233 *lac*::Tn*5*	This work
Rm7309	Rm 1021*exoR95*::Tn*5*-233*exoS** *lac*::Tn*5*	This work
Rm7312	Rm 1021*exoR95*::Tn*5*-233	This work
Rm7404	Rm1021*lac*KS1::Tn*5*-233	Jelesko and Leigh 1994
KS1	Rm1021*lac*KS1::Tn*5*	G. Walker
E. coli		
MM294A	*pro82, thi1, endA1,hsdS2*	G. Walker
MT614	MM294A *recA56*Ω6::Tn*5*	T. Finan
C2110	*polA*1, *rha, his,* Nal^r	E. Nester
Plasmids		
pRK600	pRK2013nm::Tn*9*	Finan *et al.* 1986
pRK607	pRK600 containing Tn*5*-233	de Vos *et al.* 1986
pD56*exoY*::Tn*3*HoKm	Cosmid clone of *exo* genes with *exoY-lacZ* fusion	Zhan and Leigh 1990
pM13	*exoS* cosmid	Doherty *et al.* 1988
pSP329	Broad host range cloning vector, Tc^r	E. Nester
pDO3.7	3.7-kb *Xba*I-*Bgl*II *exoS* fragment in pSP329	This work
Bacteriophage		
ϕM12	Generalized transducing phage for *R. meliloti*	Finan *et al.* 1984

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