syrM* Is Involved in the Determination of the Amount and Ratio of the Two Forms of the Acidic Exopolysaccharide EPSI in *Rhizobium meliloti

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For the invasion of root nodule cells of alfalfa by its symbiotic partner *Rhizobium meliloti*, the bacterial succinoglycan (EPSI) is required. Nitrogen starvation of *R. meliloti* results in a considerable increase of EPSI production. We have shown that the presence of nitrogen also altered the ratio of high and low molecular weight (HMW and LMW, respectively) forms of EPSI, favoring the production of LMW molecules. The transcription of two *exo* genes (*exoF*, *exoP*) was found to be under the control of combined nitrogen. In contrast, *exoH* and *exoK* were expressed independently of the nitrogen level. *nodC* and *syrM*, regulatory genes for the nitrogen control of *nod* gene expression, were shown to be involved in the regulation of EPSI production. In an *syrM* mutant both the amount and the ratio of the two main forms of EPSI were altered, compared with the wild-type strain: a reduced level of EPSI and the dominance of the LMW form in nitrogen-starved cultures, while an increased level of EPSI and the accumulation of the HMW fraction in nitrogen-containing samples was observed. An enhanced expression level of the *exoK* gene and the higher ratio of the LMW form to the total EPSI in nitrogen-free samples of the *syrM* mutant suggest a regulatory role for *syrM* in *exoK* expression and a contribution of *exoK* to the accumulation of the LMW form of EPSI.

During the formation of nitrogen-fixing root nodules, signal molecules are exchanged between the host plant alfalfa (*Medicago sativa*) and its symbiotic partner *Rhizobium meliloti*. The signal molecules are required for the control of the highly specific plant-bacterium recognition and for the coordinated interaction of the two organisms.

Molecular mechanisms acting at the early steps of nodule formation have been characterized in the *R. meliloti*-*M. sativa* symbiosis (Long 1989; Franssen et al. 1992; Schultze et al. 1994; Dénarié et al. 1996). The first signals (specific flavonoids) produced by the host plant induce the nodulation (*nod*) genes in *R. meliloti* (Mulligan and Long 1985; Peters et al. 1986), whose products are involved in the synthesis and

transport of specific lipochitooligosaccharides, the Nod factors (Lerouge et al. 1990; Schultze et al. 1992). In response to this bacterial signal, root cortical cell division is induced, resulting in the formation of the nodule structure. Bacteria invade the nodule cells through a tubular structure, the infection thread, and differentiate into nitrogen-fixing bacteroids. For the invasion of nodule cells additional molecules of signal function are required (Leigh and Coplin 1992; Kannenberg and Brewin 1994). *R. meliloti* mutants deficient in the production of the acidic exopolysaccharide (EPSI) induce empty nodules incapable of nitrogen fixation (Leigh et al. 1985). Due to the formation of abortive infection threads by the *exo* mutants, bacteria are unable to penetrate the nodule tissue (Finan et al. 1985; Leigh et al. 1987; Müller et al. 1988).

The acidic EPSI is a heteropolysaccharide composed of repeating octasaccharide subunits, each containing one galactose and seven glucose residues, carrying one acetyl, one succinyl, and one pyruvyl substituent per subunit (Åman et al. 1981). Genes required for the biosynthesis of EPSI have been identified, sequenced (Leigh et al. 1985; Long et al. 1988b; Buendia et al. 1991; Becker et al. 1993a, 1993b, 1995a; Glucksmann et al. 1993a; Müller et al. 1993), and investigated for their role in the synthesis, modification, polymerization, and export of EPSI (Glucksmann et al. 1993a, 1993b; Reuber and Walker 1993). EPSI is secreted in both high molecular weight (HMW) and low molecular weight (LMW) forms (Leigh and Lee 1988). The Fix⁺ phenotype and the nodule invasion ability of the *exo* mutants were partially restored on alfalfa host by the addition of the purified wild-type LMW fraction of EPSI; however, the HMW form was inactive (Urzainqui and Walker 1992; Battisti et al. 1992).

The *mucR* and the *expR* mutants have the ability to synthesize EPSII, a galactoglucan polymer of disaccharide subunits composed of glucose and galactose with acetyl and pyruvyl modifications (Glazebrook and Walker 1989; Zhan et al. 1989). The LMW form of EPSII produced by the *expR* mutant consisting of 15 to 20 disaccharide subunits is also able to restore the nodulation ability of an *exo* mutant (González et al. 1996), suggesting that the size of EPS molecules may be important for signal specificity.

The ratio of HMW and LMW forms of EPSI is determined and maintained by several mechanisms in *R. meliloti*. Biochemical analysis and genetic data indicate that the *exoP*,

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exoQ, and *exoT* genes are involved in the polymerization of the octasaccharide subunits and in the export of the polymer to the cell surface (Becker et al. 1993b, 1995b; Glucksmann et al. 1993b; Reuber and Walker 1993). The ExoP protein seems to be required for the polymerization of both HMW and LMW forms and for the regulation of their ratio (Becker et al. 1995b). The *exoT* and *exoQ* genes may participate in the synthesis of LMW and HMW molecules, respectively (York and Walker 1997). On the basis of sequence homologies, ExoK was proposed to be an endo-1,3-1,4- β -glycanase involved in the conversion of the HMW form to the LMW form (Glucksmann et al. 1993b; Becker et al. 1993a). Therefore, the ExoK protein with depolymerizing activity represents another factor determining the ratio of the two major EPSI components (York and Walker 1998). Recently, a second gene, *exsH*, coding for an endoglycanase, has also been shown to specifically depolymerize succinoglycan, and the contribution of ExsH to the production of LMW EPSI has been demonstrated (York and Walker 1997, 1998).

In *R. meliloti*, complex regulation mechanisms acting at both transcriptional and post-translational levels control the synthesis of EPSs. The genes *mucR* (Keller et al. 1995) and *expR* (Glazebrook and Walker 1989) have been shown to regulate the expression of *exo* and *exp* genes determining the biosynthesis of EPSI and EPSII, respectively. The product of *exoR* was shown to negatively regulate the transcription of other *exo* genes (Reed et al. 1991b). Mutations in either *exoR* or *exoS* resulted in an increased EPSI production, a mucoid phenotype, and nodulation defect in symbiosis with alfalfa (Ozga et al. 1994). In addition, ExoR was suggested to mediate the response to an environmental factor, the inhibition of EPSI synthesis by the level of ammonia. Under nitrogen-limiting conditions the synthesis of EPSI is increased in cultures of *R. meliloti* (Doherty et al. 1988). Although in *exoR* mutants the succinoglycan synthesis seemed to be insensitive to ammonia inhibition, in further experiments repression by ammonia could be detected, suggesting that an ammonia-sensing role for ExoR is unlikely (Ozga et al. 1994). Another gene, *exoX*, is involved in the post-translational regulation of EPSI production (Zhan and Leigh 1990).

Fig. 1. Total extracellular polysaccharide production by wild type (w.t.) and mutant (*ntrC*, *syrM*, *exoB*, and *exoR*) strains of *Rhizobium meliloti*. Bacterial cultures from each strain were incubated in nitrogen-free medium (-N) or in the presence of 20 mM ammonium sulfate (+N), either without luteolin (empty bars), or with 10 μ M luteolin (hatched bars). Polysaccharides obtained in two to four independent experiments were precipitated from the supernatants, hexose content of the samples was determined, and values of OD₄₈₅ per mg of protein $\times$ ml were calculated. Data of a representative experiment are presented as percentage of wild-type values (100% at -N; 120; at +N: 1.6 OD₄₈₅ per mg of protein $\times$ ml).

Earlier observations concerning the role of the *R. meliloti* gene *syrM* indicated that, in addition to its participation in *nod* gene induction, it may be involved in the regulation of late functions during nodule development. It has been shown that an *syrM* mutant of *R. meliloti* AK631 induced fewer nodules than the wild-type strain on *M. sativa* (Kondorosi et al. 1991), as well as the *syrM* mutant of *R. meliloti* 1021, either in nitrogen-free or nitrogen-supplemented medium (I. Dusha, unpublished data). This effect is not likely to be due to a lower expression level of *nod* genes, since the β -galactosidase activity expressed from a *nodABC-lacZ* fusion was at least as high in the *syrM* mutant EK217 as in the wild-type strain (Dusha and Kondorosi 1993). This suggests that *syrM* may be involved in other functions during infection and supports the data of Mulligan and Long (1989), who observed mucoid colony morphology after introducing the *syrM-nodD3-syrA* region into *R. meliloti* 1021. Tn5 insertion in the *syrM* gene or in the *syrA* locus eliminated the mucoid phenotype. On the other hand, Tn5 insertions in *exo* genes (*exoA*, *exoB*, or *exoF*) also suppressed the mucoid colony morphology. Further experiments confirmed that *syrM* may affect the expression of these genes, via positively controlling *syrA* transcription (Barnett et al. 1998).

The regulatory role of *syrM* on *nod* gene expression is established via the self-amplifying regulatory circuit of *nodD3-syrM* (Maillet et al. 1990; Kondorosi et al. 1991; Swanson et al. 1993). Thus, *syrM* participates in the nitrogen control of *nod* genes in *R. meliloti* mediated by *nodD3* and the general nitrogen regulatory system *ntr*. The role of *ntrC* (Szeto et al. 1987) as a positive regulator for *nodD3* expression has been shown earlier (Dusha and Kondorosi 1993). The level of specific flavonoids produced by the plant host (e.g., luteolin) may also influence the expression of the *nodD3-syrM* genes, since in conditions of nitrogen starvation the expression of *nodD3* is

Fig. 2. Separation of high molecular weight (HMW) and low molecular weight (LMW) fractions of the succinoglycan (EPSI) produced by the wild-type *Rhizobium meliloti* strain 1021. Bacteria were incubated under four different conditions. **A**, -N-L; **B**, -N+L; **C**, +N-L; **D**, +N+L. -N and +N: nitrogen-free medium or presence of 20 mM ammonium sulfate; -L and +L: absence and presence, respectively, of luteolin during incubation. Hexose content of the fractions of Bio-Gel A5m chromatography was determined, and values of OD₄₈₅ per 25 ml of supernatant were calculated. Two independent experiments were carried out under the four growth conditions; data of one representative experiment are shown. Bold numbers in the middle of **A-D** show total EPSI content; bold numbers at the peaks represent amount of EPSI of the separated peaks. Below these numbers: HMW and LMW fractions as percentages of total EPSI content of the sample are shown.

inducible by luteolin (Dusha et al. 1989). As was demonstrated earlier, invading rhizobia or purified Nod factors are able to specifically induce enzymes of the phenylpropanoid pathway of host plants (Estabrook and Sengupta-Gopalan 1991; Savouré et al. 1994), resulting in an increased synthesis of *nod* gene activating flavonoids (van Brussel et al. 1990; Dakora et al. 1993). This effect is assumed to be part of a positive regulation event of nodulation. Since *syrM* is a common regulatory factor for both *nod* and *exo* gene expression, the increased flavonoid levels may also affect the polysaccharide synthesis of the invading bacteria via *nodD3-syrM*. Therefore, our aim was to investigate the possible involvement of *syrM* in the control of *exo* gene expression by addressing the following questions: (i) Do *syrM* and *ntrC* contribute to the ammonia regulation of EPSI biosynthesis? (ii) Does the ammonia level influence the HMW:LMW ratio, and hence the amount of biologically active fraction of EPSI? and (iii) Does *syrM* mediate any effect of luteolin on the synthesis of EPSI?

RESULTS

Involvement of the regulatory genes *ntrC* and *syrM* in the control of EPS production by *R. meliloti* 1021.

The amount of total cetrinide precipitable extracellular polysaccharide was determined from supernatants of wild-type *R. meliloti* 1021 and two mutants, *R. meliloti* 5002 and *R. meliloti* EK217, defective in the *ntrC* and the *syrM* genes, respectively. Values obtained with cultures grown under conditions of nitrogen starvation or in the presence of 20 mM ammonium sulfate in minimal medium are compared in Figure 1. The loss of *ntrC* function resulted in a decreased amount of EPS in nitrogen-free conditions, suggesting a role for NtrC in this process. The mutation in *syrM* resulted in reduced EPS production as well. However, in the presence of ammonium sulfate and luteolin, a considerable accumulation of cetrinide precipitable material was observed in the *syrM* mutant, compared with the polysaccharide level of the wild-type strain. An *exoB* mutant blocked in EPSI and EPSII synthesis and an *exoR* mutant exhibiting derepressed EPS production were used as controls (Fig. 1). The residual polysaccharide present in the supernatant of the *exoB* mutant had been identified earlier as β -(1-2)-glucan by nuclear magnetic resonance (NMR) spectroscopy (Leigh and Lee 1988).

Thus, in nitrogen-free conditions both NtrC and SyrM play a positive regulatory role in EPSI production; however, in nitrogen-supplied cultures SyrM seems to have a negative effect. Due to the possible impact of *syrM* on the amount of EPS, and to the unexpected phenotype obtained in the presence of nitrogen and luteolin, succinoglycan production by the *syrM* mutant was further investigated and compared with that of the wild-type strain.

The amount of EPSI and the ratio of the HMW and LMW forms synthesized by wild-type *R. meliloti*.

First it was determined how the ratio of the two main (HMW and LMW) fractions of EPSI is controlled by the level of nitrogen. *R. meliloti* 1021 was grown in minimal medium supplemented as described in Materials and Methods. Lyophilized supernatants of the cultures were dissolved and chromatographed on a Bio-Gel A5m column. Fractions obtained from nitrogen-starved (Fig. 2A,B) and nitrogen-supplemented

(Fig. 2C,D) samples were assayed for carbohydrate. As expected, nitrogen limitation resulted in an at least sixfold increase in total polysaccharide production (Fig. 2A vs C). The presence of the plant inducer luteolin further increased EPSI production at both nitrogen concentrations. The effect of luteolin was even more pronounced in nitrogen-containing cultures, resulting in the production of 2.5 times more polysaccharide (Fig. 2D vs C).

In nitrogen-starved cultures the separated HMW and LMW forms represented about 60 and 40% of the total EPSI (Fig. 2A,B). In addition to the reduced amount of total polysaccharide, an altered HMW:LMW ratio was also observed in nitrogen-supplemented media. High nitrogen level favored the production of the LMW fraction: about 10:90% and 30:70% of HMW:LMW forms were detected in samples C and D, respectively (Fig. 2). To confirm that both the HMW and LMW fractions represent EPSI, samples from the peaks were collected and analyzed for hexose composition. The glucose:galactose ratio was found to be 7:1 ($\pm 10\%$ SD) characteristic of succinoglycan; therefore, the peaks of Bio-Gel A5m chromatography were identified as predominantly EPSI. These results showed that combined nitrogen strongly reduced EPSI production, particularly that of the HMW form.

syrM affects the amount of EPSI and the ratio of HMW and LMW fractions.

In the supernatant of the *syrM* mutant *R. meliloti* EK217 an altered amount and pattern of EPSI were detected. Cultures grown under the same conditions as the wild-type strain produced a lower amount of total EPSI (64% of that of the wild-type strain) in nitrogen-limited conditions (Fig. 3A,B). Surprisingly, in the presence of nitrogen more polysaccharide was synthesized by the *syrM* mutant than by the wild-type strain (117 and 207%; Fig. 3C,D). Therefore, either the repression of the total EPSI

Fig. 3. Distribution of high molecular weight (HMW) and low molecular weight (LMW) fractions of the succinoglycan (EPSI) produced by the *syrM* mutant *Rhizobium meliloti* EK217. Growth conditions and calculations are as in Figure 2. Chromatography was repeated with two (under conditions of A and B) or four (under conditions of C and D) independently grown cultures. Results of one representative experiment are shown. Percentage values below the numbers in the middle of A–D show total EPSI content of the samples as percentage of the corresponding values of the wild-type strain.

synthesis by nitrogen was lower in the mutant (71%; Fig. 3A–C) than in the wild-type strain (84%; Fig. 2A–C), or, in the luteolin-containing cultures, no repression was observed (Fig. 3B–D).

The distribution of LMW and HMW forms was found to be 10:90% (Fig. 3A,B), favoring the production of the LMW form. In contrast, the accumulation of the HMW form was observed in the presence of nitrogen (50:50% and about 75:25%; Fig. 3C,D). Therefore, under both growth conditions the distribution of the two main forms of EPSI was opposite of that found in the wild-type strain. Moreover, in the presence of luteolin EPSI forms of intermediate molecular weight

Fig. 4. Expression of *exoH-lacZ*, *exoK-lacZ*, *exoF::phoA*, and *exoP::phoA* genes in *Rhizobium meliloti* at different concentrations of nitrogen. Units of β -galactosidase and alkaline phosphatase activities were calculated as $1,000 \times OD_{420}/OD_{600} \times ml \times min$. -L and +L: absence and presence, respectively, of luteolin during growth. N: ammonium sulfate. β -Galactosidase activities were calculated from parallel samples of eight (*exoK-lacZ*) or six (*exoH-lacZ*) independent clones (SD $\leq 10\%$). Alkaline phosphatase activities of three (*exoF::phoA*) or seven (*exoP::phoA*) independent clones were determined. Values are presented as percentage of the activity determined in samples incubated without luteolin and nitrogen. 100% *exoF::phoA* activity: 101 ± 24 ; 100% *exoP::phoA* activity: $59 \pm 12 OD_{420}/OD_{600} \times ml \times min$.

Fig. 5. Effect of *syrM* mutation on expression of *exoK-lacZ*, *exoH-lacZ*, and *exoP::phoA* genes. Enzyme activities determined in the wild-type background are shown as empty bars (data are taken from Figure 4). Enzyme activities measured in the *syrM* mutant are represented by hatched bars. β -Galactosidase activities were calculated from parallel samples of 10 (*exoK-lacZ*) or eight (*exoH-lacZ*) independent clones (SD $\leq 10\%$). Alkaline phosphatase activities were determined from 11 independent clones and are shown as percentage of the activity of the wild-type strain incubated without nitrogen and luteolin. 100% *exoP::phoA* activity: $59 \pm 12 OD_{420}/OD_{600} \times ml \times min$. Other symbols are identical to those of Figure 4.

appeared, resulting in the absence of the two distinct peaks in the chromatogram (Fig. 3D). The hexose composition of the fractions was determined similarly to that of the wild-type strain. Where no separate peaks appeared (Fig. 3D), three samples were collected, from fractions 13 to 20 (HMW), 21 to 28 (intermediate), and 29 to 38 (LMW). In each case a glucose:galactose ratio characteristic of EPSI was obtained.

Thus, *syrM* is indeed required for the production of both the HMW (-N) and LMW (+N) fractions, and participates in the mediation of the repression of EPSI synthesis by nitrogen.

Nitrogen dependence of *exo* gene expression.

To examine whether the reduced production of EPSI is due to the regulation of specific *exo* genes by nitrogen, the expression of several *exo* genes was determined under various growth conditions. The product of *exoF* is required for the addition of the first sugar, galactose, to the lipid carrier, thus representing an early step in the biosynthesis of succinoglycan subunits (Reuber and Walker 1993). Another gene, *exoP*, codes for a protein involved in functions after subunit assembly, since ExoP seems to participate in polymerization and/or export of the polymer (Reuber and Walker 1993; Becker et al. 1993b; Glucksmann et al. 1993b). The expression of *exoF* and *exoP* was examined by measuring alkaline phosphatase activities of *exoF::phoA* and *exoP::phoA* fusions. When the *R. meliloti* strain 8268 carrying *exoF::phoA* was grown with 20 mM ammonium sulfate as nitrogen source, the expression level of *exoF* was about 50% lower than in samples grown at limiting concentrations of ammonium sulfate (Fig. 4A). The expression of *exoP::phoA* in the *R. meliloti* strain 8272 was also reduced by increased nitrogen concentration. However, the addition of luteolin resulted in a phosphatase activity comparable to that measured at low nitrogen level (Fig. 4B).

The *exoP* gene is a member of the *exoHKLAMONP* gene cluster (Reuber et al. 1991; Becker et al. 1993b). It has been shown that a strong promoter upstream of *exoH* is able to direct the transcription of the whole cluster (Becker et al. 1993a). In addition, weak promoters upstream of other members of the cluster (e.g., *exoK* and *exoP*) have been identified. Using *exoH-lacZ* and *exoK-lacZ* fusions, we examined whether the strong promoter of *exoH* and the weak promoter of *exoK* are also under nitrogen control. In both cases no effect of nitrogen on transcription could be demonstrated (Fig. 4C,D). These results suggest that specific steps of the EPSI biosynthesis are controlled by nitrogen: *exoF* and *exoP* are involved in the down-regulation of EPSI production by nitrogen.

Are the *exoK* and *exoP* genes involved in the control of the distribution of HMW and LMW forms of EPSI by *syrM*?

Since in the *syrM* mutant an altered ratio of LMW and HMW EPSI was observed, we examined the possible effect of the *syrM* mutation on the expression of genes required for the polymerization or specific cleavage of EPSI. The two genes selected for these studies were *exoP*, involved in succinoglycan polymerization, and *exoK*, coding for an endoglycanase with depolymerizing activity (Reuber and Walker 1993; Glucksmann et al. 1993b; Becker et al. 1993b; York and Walker 1997).

In nitrogen-limited cultures of the *syrM* mutant the expression of the *exoK-lacZ* fusion (*R. meliloti* OB82) was two to three times higher than in the wild-type strain (Fig. 5A). At nonlimiting nitrogen concentrations, however, β -galactosidase

activity was only 30 to 50% higher. To test whether this effect on the expression of *exoK* was due to transcription control acting at the strong promoter located upstream of *exoH*, the expression of *exoH-lacZ* was also tested in the *syrM* mutant (*R. meliloti* OB83). Only a 30 to 40% increase of β -galactosidase activity was found, compared with the wild type control (Fig. 5B). Therefore, the regulation observed for the *exoK* expression is probably due to a mechanism specifically affecting the weak promoter upstream of *exoK*. These data suggest that the increased level of ExoK protein under nitrogen-limited conditions might contribute to the accumulation of the LMW EPSI fraction observed in the *syrM* mutant, resulting in the 10 to 90% distribution of HMW-LMW forms (Fig. 3A,B).

The EPSI production of the *exoK* mutant *R. meliloti* 8445 was determined from the supernatants of cultures grown under the same conditions as the wild-type strain. In nitrogen-starved samples a more than 50% decrease in the total EPSI level was observed (Fig. 6A1,B1). Although the strain is deficient in the depolymerizing activity of ExoK, 60 to 70% of the EPSI was represented by the LMW form. Both the biosynthetic activities of ExoP and ExoT and the presence of *exsH* coding for an alternative endo-1,3-1,4- β -glycanase (York and Walker 1997) may be responsible for the formation of LMW molecules in the *exoK* mutant. In nitrogen-supplied cultures the ratio of the two forms resembled that of the wild-type strain, although at a lower total EPSI level (Fig. 6C1,D1).

Since in the presence of nitrogen the *exoK* gene is expressed at the same level in the wild-type strain as in the *syrM* mutant, and these strains produce an opposite distribution of the two EPSI forms, we supposed that other protein(s) participating in the polymerization of succinoglycan may be responsible for the accumulation of the HMW form (Fig. 3C,D vs Fig. 2C,D). As a candidate for such function, the expression of *exoP* was examined in the *syrM* mutant.

Fig. 6. Separation of high molecular weight (HMW) and low molecular weight (LMW) polymers of the succinoglycan (EPSI) obtained from the supernatants of (A1–D1) *Rhizobium meliloti* 8445 and (A2–D2) *Rhizobium meliloti* 8272. Chromatography was repeated from (A1–D1) four or (A2–D2) two independently grown cultures. Results of representative experiments are shown. Growth conditions and symbols are as in Figure 2.

Under any of the growth conditions tested, no HMW fraction of EPSI could be detected in the strain carrying *exoP::phoA* (Fig. 6A2–D2) in accordance with the proposed role of ExoP in the polymerization of the repeating octasaccharide units. The amount of LMW EPSI was still about 40% of that of the wild-type control, indicating the involvement of other genes (e.g., *exoT*) with proposed polymerization activity in the synthesis of EPSI polymers. To examine whether the elevated level of the HMW form (50 to 75%; Fig. 3C,D) of EPSI in nitrogen-supplemented cultures of the *syrM* mutant could be due to the increased expression of *exoP*, alkaline phosphatase activity of *exoP::phoA* fusion was determined (*R. meliloti* OB84). EPSI produced by the wild-type cells under these conditions consisted of 70 to 80% LMW form (Fig. 2C,D). However, in the *syrM* mutant alkaline phosphatase activities were similar (–L) or lower (+L) than in the wild-type cells (Fig. 5C), suggesting that the control of *exoP* expression does not contribute to the observed effect.

DISCUSSION

In this paper we have shown that the *syrM* gene of *R. meliloti* 1021, described earlier as a regulator for *nod* gene expression and an activator for the *syrA* gene, is involved in the determination of the ratio of LMW and HMW forms of EPSI, and that its effect depends on both nitrogen availability and the presence of the plant flavonoid luteolin (Fig. 7).

Nitrogen level controls the expression of several *exo* genes.

It was published earlier that the presence of ammonia inhibits the synthesis of EPSI (Leigh et al. 1985). In conditions

Fig. 7. Model for involvement of *syrM* in the regulation of succinoglycan (EPSI) production of *Rhizobium meliloti* 1021. *syrM* forms a self-amplifying regulatory circuit with the *nodD3* gene (Maillet et al. 1990; Kondorosi et al. 1991; Swanson et al. 1993), and affects the level of EPSI production, probably via positively controlling *syrA* transcription (Barnett et al. 1998). The signal of combined nitrogen level, mediated by the *ntr* system to *nodD3* gene (Dusha and Kondorosi 1993), may modulate the rate of EPSI synthesis via *nodD3-syrM*: the expression of *exoF* and *exoP* is under the control of ammonia (this work). Distribution of high molecular weight (HMW) and low molecular weight (LMW) forms of EPSI is also influenced by *syrM*, probably by determining the polymerization mode of ExoP (ExoP_H and ExoP_L representing the two conformations of the enzyme) (this work). ExoQ and ExoT contribute to the polymerization of succinoglycan octamer units to form HMW and LMW molecules, respectively (York and Walker 1997) while *exoK*, one of the genes coding for a depolymerizing enzyme, is negatively regulated by *syrM* in nitrogen-starved conditions (this work). Presence of the plant flavonoid luteolin, an inducer of *nodD3* (Dusha et al. 1989), brings about an enhanced succinoglycan production, and a higher expression level of *exoF* and *exoP* (this work).

of nitrogen starvation, a five- to 20-fold increase in the amount of EPSI can be detected (Doherty et al. 1988, Reed et al. 1991a; Reed and Walker 1991). The production of the second EPS, EPSII, however, is not inducible under nitrogen-limiting conditions (Zhan et al. 1991).

We have shown that the transcription of two *exo* genes, *exoF* and *exoP*, is under the control of nitrogen. In contrast, two other genes, *exoH* and *exoK*, were expressed independently of the nitrogen concentration. Furthermore, our results indicate that genes involved in nitrogen regulatory processes (*ntrC*) or in nitrogen control of Nod factor production (*syrM*) are also required for the synthesis of EPSI. In the absence of these gene products a lower level of total extracellular polysaccharide was found in conditions of nitrogen limitation. The observed effect of *syrM* is in agreement with the results of Barnett et al. (1998), who demonstrated that the amount of EPSI is under the control of the *syrM*-activated *syrA* gene.

Nitrogen level affects the distribution of HMW and LMW polymers of EPSI, and *syrM* is involved in the determination of their ratio.

We have demonstrated that the amount of HMW and LMW fractions of EPSI produced by the wild-type *R. meliloti* 1021 in nitrogen-limited conditions is about 60 and 40%, respectively. By the use of a similar method for separation, the same ratio was obtained by Becker et al. (1995b). Increasing the concentration of nitrogen up to 20 mM in the medium, we observed not only a reduction of the amount of EPSI to 15%, but also the alteration of the ratio of fractions: 14% HMW and 86% LMW forms appeared in the supernatants of the cultures. These results could be explained as an effect of the lower biosynthetic rate in the presence of nitrogen, since—as suggested by Reed et al. (1991a)—under such conditions the formation of the LMW form is favored.

The assumption that the increased ratio of LMW to the total EPSI in nitrogen-supplied samples is due to the adaptation of the cells to the conditions of higher osmolarity can probably be excluded. As was shown by Breedveld et al. (1990), the production of succinoglycan was enhanced by increasing osmotic pressure (e.g., in 200 mM NaCl). Under those conditions the accumulation of HMW forms was preferred. In contrast, in the *syrM* mutant, a decreased production of EPSI and a higher proportion of LMW form to the total EPSI was demonstrated at increased salt concentration (20 mM).

In the absence of a functional *syrM* both the amount of EPSI and the ratio of the two main forms were affected: the EPSI content was lower in nitrogen-starved samples and higher in nitrogen-supplied samples, compared with that of the wild-type cells. Moreover, the ratio of the two forms was also the opposite of that found in wild-type cells: in nitrogen-containing samples the accumulation of the HMW form, and in nitrogen limitation the dominance of the LMW form was found. However, the shift in favor of the LMW oligosaccharide production cannot be explained as a consequence of a considerable decrease in EPSI synthesis, as was suggested by Reed et al. (1991a), since the total succinoglycan content was still 65% of that of the wild-type cells.

Under nitrogen-limiting conditions *syrM* mutation results in an increased *exoK* expression.

The size distribution of the succinoglycan polymer may depend on the action of several gene products. To examine

whether these genes could be regulated by *syrM*, and whether their altered control could explain the changes in the EPSI size distribution, the expression level of *exoK* and *exoP* was determined in an *syrM* mutant. The *exoK* expression was found to be two to three times higher than in the wild-type strain. When the cultures were supplied with nitrogen, this effect was not observed, in accordance with the detection of a much lower ratio for the LMW form. The production of less LMW succinoglycan was not due to nitrogen repression of the *exoK* gene expression, since in the nitrogen-supplied *syrM* mutant the expression of *exoK* was similar to that of the wild-type cells.

The regulation by *syrM* seems to affect primarily the weak promoter present upstream of *exoK*. As a control, *exoH* expression was also examined in the *syrM* mutant, but only a 25 to 35% increase in its level was found. This may indicate that the strong promoter present upstream of *exoH*, responsible also for *exoK* expression, is not under the strict control of *syrM*. The results of Urzainqui and Walker (1992) also support our observations. When a plasmid carrying the *exoHKL* genes was introduced into the wild-type *R. meliloti* strain 1021, LMW succinoglycan was synthesized at an elevated level (three times more than in the wild-type strain). By the use of Tn5 mutations in the three coding regions, it was shown that the production of more LMW form was due to the overexpression of *exoK*. Although the *exoH* mutation completely eliminates the production of the LMW form (Leigh and Lee 1988), this seems to be due to the low degree of susceptibility of the non-succinylated EPSI to cleavage by ExoK, and not to a polar effect of *exoH* mutation on *exoK* expression (York and Walker 1998). Therefore, we may conclude that either the introduction of *exoK* in multiple copies, or a mutation in *syrM* allowing a higher expression level of *exoK*, may result in the appearance of more LMW molecules in the supernatant.

Another gene, *exoP*, that may influence the ratio of the two forms was expressed at similar levels in the *syrM* mutant and the wild-type strain, except in nitrogen- and luteolin-containing medium, where, in contrast to the wild-type cells, *exoP* remained nitrogen repressed (Fig. 5C). Therefore, the enhanced amount of total EPSI production and the shift of the ratio toward the formation of HMW forms (Fig. 3D) were not the result of a higher expression level of *exoP*. Another explanation could be that alterations in the polymerization and termination mode of ExoP activity are responsible for the observed effect in the *syrM* mutant. Since ExoP is involved in the production of both EPSI forms, the protein was proposed to exist in two conformational stages, one promoting polymerization and the other one allowing termination of polysaccharide chains (Becker et al. 1995b). The formation of EPSI as well as the *O*-polysaccharide chains of lipopolysaccharides (Batchelor et al. 1992; Bastin et al. 1993; Morona et al. 1995) is characterized by a bimodal distribution of chain length. In *Escherichia coli* and *Salmonella* and *Shigella* strains, this is maintained by chain-length determinants (Cld or Rol proteins) that, acting as molecular clocks, control the activity of polymerizing enzymes by switching between a polymerization and termination mode (Batchelor et al. 1991; Bastin et al. 1993). Mutants affected in *rol* or *cld* regulatory functions produce deregulated chain-length distribution, similar to the pattern of EPSI obtained in the *syrM* mutant (Fig. 3D). By analogy, we suggest the involvement of an as yet unidentified regulatory protein that may act under the direct or indirect control of *syrM* in determining the mode of

ExoP activity. We assume that the increased and deregulated EPSI production of the *syrM* mutant in the presence of nitrogen may be due to the lack of nitrogen repression, and an altered regulation of ExoP conformation. In addition, since the synthesis of the LMW molecules is not enhanced as a response to luteolin, this may further induce EPSI production by a feedback effect on the activity of ExoP.

EPSI synthesis exhibits an *syrM*-dependent response to the plant flavonoid luteolin.

The presence of luteolin resulted in an elevated succinoglycan production in both the wild-type and *syrM* mutant strains. The increase of the EPSI level was more pronounced in nitrogen-repressed cultures; moreover, an enhancement of the LMW fraction of EPSI was also observed. It was shown earlier by Reuhs et al. (1995) that alfalfa root exudate elicited an elevated synthesis of the HMW capsular polysaccharide of *R. meliloti* AK631. In *R. fredii*, genistein was found to increase extracellular polysaccharide production (Dunn et al. 1992). In this paper we demonstrate the involvement of *syrM* in the luteolin-enhanced EPSI synthesis, since, in contrast to the wild-type strain, a mutant deficient in *SyrM* is not able to alter the HMW:LMW ratio in favor of the LMW form (see above). However, the presence of luteolin may also have a direct effect on EPSI synthesis. As was shown earlier, flavonoid molecules accumulate in the inner membrane of *Rhizobium* spp. (Recourt et al. 1989). Since the enzymes for EPSI polymerization and transport are membrane-localized proteins, the perturbed membrane structure may induce changes in their conformation.

The effect of luteolin was also observed at the level of gene expression. Nitrogen regulated genes (*exoF* and *exoP*) exhibited an enhanced expression in the presence of luteolin, in contrast to the nonregulated *exoH* and *exoK*. The role of *syrM* is demonstrated by a higher expression level of *exoK* in the nitrogen-starved *syrM* mutant strain. This effect is further enhanced in the presence of luteolin. It is worth mentioning that the expression of the *exoP* gene was not repressed by nitrogen in the presence of luteolin in the wild-type cells, while in the *syrM* mutant this "escape" from nitrogen repression was not observed. The reason for the lack of nitrogen repression of *exoP* in the presence of luteolin is unknown. It seems, however, that *syrM* is also required for this function.

We have speculated on the biological significance of the repression of EPSI biosynthesis by nitrogen and the control of the ratio of the two forms. The production of EPSI as a highly energy-consuming process is maintained at a low level. In stress conditions, such as nitrogen starvation or high osmolarity environment, more EPSI is required for the protection of the cells. Our data suggest that *SyrM*, involved in the determination of both the amount of EPSI and the ratio of the two forms, contributes to the adjustment of the optimum level and polymer ratio. In nitrogen-free conditions, bacteria deficient in *SyrM* are not able to increase the amount of total EPSI (only that of the LMW form) to the wild-type level. Only nitrogen-supplied and luteolin-induced cells produce EPSI in amounts comparable to that of the wild-type strain; however, under such conditions the synthesis of HMW molecules seems either to be deregulated, or an enhanced degradation may result in the formation of molecules with intermediate chain length. The establishment of the symbiotic relationship may also be

considered an adaptation to a new environment with altered requirements for different EPSI forms. In a nitrogen-containing environment the nodulation of the host plant by bacteria is reduced. The decreased production of EPSI meets the requirements of these conditions. The relative accumulation of the LMW form may be important for providing the biological signal function toward the host plant in conditions of reduced EPSI biosynthetic rate. However, the presence of LMW molecules may not be the only requirement for a successful invasion. The *syrM* mutant produces a higher level of LMW EPSI, but exhibits a delayed nodulation phenotype in plant tests. The delayed nodulation could be explained by a difference in Nod factor composition, or by the involvement of *syrM* in further, yet unknown functions. Finally, the HMW fraction may also have an important role, e.g., in protecting the bacteria against the plant defense mechanism, or may serve as an osmoprotectant.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Strains and plasmids are listed in Table 1.

Media and growth conditions.

E. coli strains were grown in LB medium at 37°C (Maniatis et al. 1982). *R. meliloti* strains were cultured at 34°C in complete medium as described previously (Kondorosi et al. 1984), or in nitrogen-free M9 (Miller 1972) or GTS (Kiss et al. 1979) medium supplemented with nitrogen source as indicated. Antibiotics were added at the following concentrations (µg/ml): for *R. meliloti*—streptomycin 200, kanamycin 200, tetracycline 15, gentamicin 200, chloramphenicol 60; for *E. coli*—kanamycin 50, tetracycline 10, chloramphenicol 30, gentamicin 10, ampicillin 50.

DNA manipulations and bacterial crosses.

Preparation of plasmid DNA, digestions with restriction enzymes, agarose gel electrophoresis, fragment isolation, cloning procedures, and transformation of *E. coli* cells were performed according to the protocols described by Maniatis et al. (1982).

Derivatives of plasmid pRK290 were transferred from *E. coli* to *R. meliloti* by conjugation in triparental matings (Ditta et al. 1980). For homogenotization, pBO7 (this work), a derivative of pVK101 (Knauf and Nester 1982) was introduced into *R. meliloti*.

Construction of *syrM* mutant strains carrying *exoP::phoA*, *exoK-lacZ*, or *exoH-lacZ* genomic fusions.

The plasmid pNID6 carrying the *syrM-nodD3* region on a 7.2-kb *Bam*HI fragment (Györgypál et al. 1988) was used to isolate the *syrM* gene on a 3.5-kb *Bam*HI-*Cla*I fragment. This region was recombined in the mobilizable plasmid pPP375, resulting in the plasmid pBO19. A 1.4-kb *Hind*III fragment containing the chloramphenicol resistance gene with its promoter was isolated from the pBR322::Tn5-CM construct (Sasakawa and Yoshikawa 1987), and ligated into the single *Xba*I site of the *syrM* gene in pBO19. The recombinant plasmid pBO20 thus obtained was used to transform *E. coli*, then was conjugated into *R. meliloti* 1021 by triparental mating. Since a *Cm*^r gene was applied to construct mutation in *syrM*, the plasmid pPH1JI (Beringer et al. 1978) also carrying a *Cm*^r gene was not suitable for homogenotization. In addition, *R. meliloti* H1a and H2a strains were resistant for gentamicin, another marker of pPH1JI. Therefore, the *Sma*I fragment within the *Tc*^r gene of the cosmid pVK101 was deleted (to avoid recombination at the *Tc*^r gene also present on pBO20, the recombinant derivative of pRK290). The resulting pVK101 derivative, pBO7, was conjugated into *R. meliloti* 1021(pBO20).

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference/source
<i>Escherichia coli</i>		
HB101	<i>hsdS, hsdM, pro, leu, thi, gal, lacY, recA, str</i>	Boyer and Rouland-Dussoix 1969
<i>Rhizobium meliloti</i>		
<i>R. meliloti</i> 1021	Wild type, Sm ^r derivative of SU47	F. M. Ausubel
<i>R. meliloti</i> 5002	<i>R. meliloti</i> 1021, <i>ntnC::Tn5</i>	Szeto et al. 1987
<i>R. meliloti</i> EK217	<i>R. meliloti</i> 1021, <i>syrM::Tn5</i>	Kondorosi et al. 1991
<i>R. meliloti</i> AT19	<i>R. meliloti</i> 1021, <i>exoB::Tn5</i>	A. Kereszt
<i>R. meliloti</i> 7095	<i>R. meliloti</i> 1021, <i>exoR95::Tn5</i>	Doherty et al. 1988
<i>R. meliloti</i> 8002	<i>R. meliloti</i> 1021, <i>Pho</i> ⁻	Long et al. 1988a
<i>R. meliloti</i> 8272	<i>R. meliloti</i> 8002, <i>exoP272::TnphoA</i>	Reuber et al. 1991
<i>R. meliloti</i> 8268	<i>R. meliloti</i> 8002, <i>exoF268::TnphoA</i>	Reuber et al. 1991
<i>R. meliloti</i> H1a	<i>R. meliloti</i> 2011, <i>exoH-lacZ</i>	Becker et al. 1993b
<i>R. meliloti</i> H2a	<i>R. meliloti</i> 2011, <i>exoK-lacZ</i>	Becker et al. 1993b
<i>R. meliloti</i> 8445	<i>R. meliloti</i> 1021, <i>exoK445::Tn5</i>	Long et al. 1988b
<i>R. meliloti</i> OB81	<i>R. meliloti</i> 1021, <i>syrM::Cm</i> ^r	This work
<i>R. meliloti</i> OB82	<i>R. meliloti</i> H2a, <i>syrM::Cm</i> ^r	This work
<i>R. meliloti</i> OB83	<i>R. meliloti</i> H1a, <i>syrM::Cm</i> ^r	This work
<i>R. meliloti</i> OB84	<i>R. meliloti</i> 8272, <i>syrM::Cm</i> ^r	This work
Plasmids and phage		
pBR322	Ampicillin and tetracycline resistant	Bolivar et al. 1977
pBR322::Tn5-CM	Tn5 derivative carrying the <i>Cm</i> ^r gene in pBR322	Sasakawa and Yoshikawa 1987
pRK290	Tetracycline resistant, IncP	Ditta et al. 1980
pPP375	pRK290 containing multiple cloning sites inserted into the single <i>Eco</i> RI site	P. Putnoky
pVK101	Kanamycin and tetracycline resistant, IncP	Knauf and Nester 1982
pNID6	7.2-kb fragment carrying <i>nodD3-syrM</i> in pRK290	Györgypál et al. 1988
pBO7	pVK101 derivative: a <i>Sma</i> I fragment with the <i>Tc</i> ^r gene was deleted	This work
phage M12		Finan et al. 1984

for selection of mutants carrying the *syrm::Cm^r* integrated into the genome. *R. meliloti* *exoP::phoA*, *exoK-lacZ*, and *exoH-lacZ* mutants also carrying the *syrm::Cm^r* region were then obtained by transduction with M12 phage and the following recipient strains: *R. meliloti* 8272, *R. meliloti* H2a, and *R. meliloti* H1a. The integration of *syrm::Cm^r* into the genome of both the wild-type and the mutant strains was confirmed by Southern hybridizations and polymerase chain reaction (PCR) amplifications with the following primers: 5'-TATCGGCA-CATTACTCAAGC-3' and 5'-TTCGCCACGCAGCCAGC-GATGC-3'.

β-Galactosidase measurement.

R. meliloti strains grown in complete medium were pelleted and washed with nitrogen-free GTS (GTS-N) medium. Samples were inoculated to obtain an optical density of 0.1 (at 600 nm) in GTS-N medium, and the cultures were supplemented with low (3 mM) or high (50 mM) concentrations of ammonium sulfate as nitrogen source. Luteolin (10 μM) was added where indicated. Bacteria were grown to optical density of 0.6 to 0.8, and β-galactosidase activity was determined as described by Miller (1972).

Alkaline phosphatase assay.

R. meliloti strains were precultured in complete medium, pelleted, and washed with nitrogen-free 1× Tris-N-P medium (containing 0.1 M Tris-HCl, pH 7.5, 0.5 mM KCl, 1 mM MgCl₂, 1 mM NaCl, 2 mM Na₂SO₄, 0.1 mM KH₂PO₄, 3 μM FeCl₃, 1 mM MgSO₄, 0.5 μg of biotin per ml, and 0.2% sucrose). The samples were diluted to obtain an optical density of 0.2 (at 600 nm) in 1× Tris-N-P medium, and ammonium sulfate was added at low (3 mM) or high (20 mM) concentrations. Luteolin was used at 10 μM final concentration where indicated. Bacteria were incubated for 72 to 120 h, and alkaline phosphatase activity was measured in 24-h intervals according to Brickman and Beckwith (1975).

EPSI production of *R. meliloti* strains.

Bacteria were grown in complete medium (supplemented with antibiotics) at 34°C to an optical density (600 nm) of 0.5. From each culture four samples of 20-ml volume were centrifuged and washed with nitrogen-free M9 medium (M9-N), then resuspended to get the same optical density in one of the following media: (i) M9-N; (ii) the same as (i) supplemented with 10 μM luteolin; (iii) M9-N+20 mM ammonium sulfate; and (iv) the same as (iii) supplemented with luteolin. In each case MgSO₄, biotin, and sucrose were also added at final concentrations of 1 mM, 0.5 μg/ml, and 0.2%, respectively. The cultures were incubated for 40 h at 34°C in a rotary shaker. Ten milliliters of each sample was used for EPS preparation. After centrifugation, 0.3 volume of hexadecyltrimethylammonium bromide (cetrimide) was added to the supernatant according to Leigh et al. (1985). The precipitate was dissolved in 10% NaCl and the hexose content was determined by the phenol-sulfuric acid method (Dubois et al. 1956). Samples with high hexose content were diluted to obtain an optical density (at 485 nm) between 0.1 and 0.4 where the calibration curve made with glucose as standard changed linearly in function of the sugar concentration. Another 10 ml of the cultures was used to determine the total cell protein content. After the cells were lysed with 10% NaOH, protein concentra-

tion was measured with the Bio-Rad (Richmond, CA) protein microassay and bovine serum albumin as standard.

EPSI isolation and separation.

R. meliloti strains were grown at 34°C in complete medium to an optical density of 0.5 at 600 nm. Four samples of 250 ml were centrifuged, washed with M9-N medium, and resuspended in the four different media described above. After 40 h of incubation at 34°C in a rotary shaker, bacteria were removed by centrifugation, and the supernatants of the cultures were lyophilized. For the separation of the LMW and HMW forms of EPSI, the lyophilized samples were dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl, and chromatographed on a Bio-Gel A5m column (2.5 × 56 cm) as described by Leigh and Lee (1988). Fractions of 6.8 ml were collected and measured for hexose content by the phenol-sulfuric acid method. Since fractions containing the LMW form of EPSI partially overlapped with the peak of sucrose present also in the lyophilized supernatant as non-utilized carbon source, samples from LMW fractions were precipitated by 10 volumes of ethanol, centrifuged, dried, and dissolved in water, then measured for the hexose content. Total EPSI content was calculated by summarizing the amounts of polysaccharide represented by the two peaks (peak areas). The amounts of HMW and LMW peak areas were expressed as percentages of the total EPSI content. To characterize the reproducibility of the data obtained in independent experiments, the variability (SD) of the amount of total EPSI (±6 to 20%) and of the peak areas (±4 to 8% for the wild-type strain and ±1.5 to 7.8% for the *syrm* and *exoK* mutants) was calculated.

Analysis of the hexose composition of EPSI fractions.

The method of Köplin et al. (1993) was used with modifications. Polysaccharide samples were hydrolyzed by 2 M trifluoroacetic acid at 100°C. The excess acid was extracted with chloroform, the layer containing monosaccharides was dried under vacuum, and the samples were dissolved in water. The glucose and galactose content was examined by isocratic high-pressure liquid chromatography (HPLC) with a Bio-Rad Aminex HPX-87 C column and refractive index detector (Bio-Rad model 1755).

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