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Environmental Regulation of Exopolysaccharide Production in *Sinorhizobium meliloti*

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Exopolysaccharide production by *Sinorhizobium meliloti* is required for invasion of root nodules on alfalfa and successful establishment of a nitrogen-fixing symbiosis between the two partners. *S. meliloti* wild-type strain Rm1021 requires production of either succinoglycan, a polymer of repeating octasaccharide subunits, or EPS II, an exopolysaccharide of repeating dimer subunits. The reason for the production of two functional exopolysaccharides is not clear. Earlier reports suggested that low-phosphate conditions stimulate the production of EPS II in Rm1021. We found that phosphate concentrations determine which exopolysaccharide is produced by *S. meliloti*. The low-phosphate conditions normally found in the soil (1 to 10 μM) stimulate EPS II production, while the high-phosphate conditions inside the nodule (20 to 100 mM) block EPS II synthesis and induce the production of succinoglycan. Interestingly, the EPS II produced by *S. meliloti* in low-phosphate conditions does not allow the invasion of alfalfa nodules. We propose that this invasion phenotype is due to the lack of the active molecular weight fraction of EPS II required for nodule invasion. An analysis of the function of PhoB in this differential exopolysaccharide production is presented.

The soil bacterium *Sinorhizobium meliloti* (*Rhizobium meliloti*) fixes nitrogen in symbiotic association with the leguminous plant *Medicago sativa* (alfalfa). Molecular analyses have revealed that early steps in the establishment of this symbiosis, including attraction of the bacteria to the plant and formation of the plant nodule, depend upon an exchange of small signaling molecules between the two partners (12, 27, 28). Bacterial invasion of root nodules requires exopolysaccharide production by *S. meliloti* (25), although the function of these exopolysaccharides in invasion is not yet clear.

S. meliloti wild-type strain Rm1021 produces succinoglycan (26), a well-characterized exopolysaccharide polymer of repeating octasaccharide subunits. Each subunit consists of one galactose and seven glucose residues and is decorated with a succinyl, an acetyl, and a pyruvyl modification (2, 36, 37). Mutants of Rm1021 that fail to synthesize succinoglycan (*exo* mutants) form empty nodules that are devoid of bacteria and are unable to fix nitrogen (24, 25). A 25-kb cluster of *exo* genes has been identified on the second symbiotic megaplasmid (8, 9, 16, 17, 24, 35) that is required for the production of succinoglycan (14, 22, 25), and biosynthetic roles have been assigned to most of the gene products (38). A tetramer fraction of succinoglycan was originally assigned as the active species in nodule invasion (7, 43), but recent evidence suggests that the active fraction is a trimer of the octasaccharide subunit (19).

Rm1021 also has the capacity to make a second distinct exopolysaccharide, designated EPS II (15), which is a polymer of repeating disaccharide subunits. Subunits of EPS II consist of an acetylated glucose connected to a pyruvylated galactose residue (15, 21). A 32-kb cluster of *exp* genes (distinct from the *exo* genes) on the second symbiotic megaplasmid (15) is responsible for the synthesis of EPS II. EPS II production by the wild-type strain Rm1021 has been previously characterized as cryptic (15). EPS II is produced at extremely low levels by this strain, but production is greatly increased in derivatives carrying the *expR101* mutation, which results in overexpression of the *exp* genes. The *expR101*-mediated synthesis of EPS II was shown to suppress the symbiotic defects of succinoglycan-deficient strains on alfalfa (15). Further analysis revealed that a low-molecular-weight fraction, consisting of 15 to 20 disaccharide subunits of EPS II, efficiently rescued nodule invasion in an exopolysaccharide-deficient strain (18), suggesting that low-molecular-weight EPS II may act as a symbiotic signal during infection.

The reason that *S. meliloti* is capable of producing two functional exopolysaccharides and how their production is regulated are not yet clear. It has been shown that EPS II production in wild-type Rm1021 increases in conditions of phosphate limitation (44). This observation provides some interesting insight when the two environments that *Sinorhizobium* may encounter (the soil and inside the nodule) are considered. The concentration of available phosphate in the soil is very low (typically 1 to 10 μM) (11). The phosphate concentrations inside plant tissues are very high (10 to 20 mM) (11). In addition, legumes that obtain nitrogen from symbiosis require higher levels of phosphate for optimal growth than plants that receive nitrogen from fertilizers (23). It has also been suggested that the concentration of phosphate inside the nodule is much higher than any other part of the plant (up to 100 mM) (23).

This report shows how phosphate concentration determines which exopolysaccharides are produced by *S. meliloti*. Low phosphate concentrations stimulate EPS II production, while high phosphate concentrations dramatically increase succinoglycan production. We show that the EPS II produced by Rm1021 in low-phosphate conditions does not allow nodule invasion to occur. We also observe that the *phoB* gene is required for the production of EPS II in low-phosphate conditions. We propose that phosphate concentration serves as a signal to regulate which exopolysaccharide is produced according to the requirements of its environment.

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MATERIALS AND METHODS

Bacterial strains, media, and genetic techniques. The strains used in this study are listed in Table 1. The construction of strains by general transduction has been

TABLE 1. Bacterial strains used in this work

Strain	Relevant characteristics	Reference or Source
Rm1021	SU47 *str-21*	25
Rm8501	Rm1021 *lac* mutant	15
Rm7210	Rm1021, *exoY210*::Tn*5-233*	25
Rm10002	Rm1021, *expA3*::Tn*5*	15
Rm11003	Rm1021, *exoY210*::Tn*5-233*, *expA3*::Tn*5*	This work
Rm8530	Rm1021, *expR101*	G. C. Walker
Rm9020	Rm1021, *expR101*, *exoY210*::Tn*5-132*	G. C. Walker
Rm11002	Rm1021, *expR101*, *expA3*::Tn*5*	This work
Rm9001	Rm1021, *mucR*::Tn*5-233*, *exoY210*::Tn*5*	18
RmH615	Rm1021, *lac* mutant, Ω*phoB3*::Tn*5*	4
Rm11000	RmH615, *exoY-GY*-Tn*3hohokm*	This work
Rm11001	RmH615, *expA404*-Tn*3hohokm*	This work
RmAR9007	Rm1021, *exoY-GY*-Tn*3hohokm*	G. C. Walker
RmAR1001	Rm1021, *expA404*-Tn*3hohokm*	G. C. Walker

previously described (13). Overnight cultures were grown in Luria-Bertani broth supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (LB-MC) and inoculated (1:200 dilution) into MOPS-MGS media modified for phosphate concentration. Modified MOPS-MGS minimal medium (44) consisted of 50 mM MOPS (morpholine propane sulfonic acid; pH 7.4), 55 mM mannitol, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 19 mM glutamic acid, and 0.004 mM biotin. Either 0 or 0.1 mM (low-phosphate) or 20, 100, 150, or 200 mM (high-phosphate) K_2HPO_4 was added. Due to osmolarity considerations, 50 mM MOPS was omitted from the 150 and 200 mM K_2HPO_4 liquid media. To provide buffering capacity and the correct pH, these media were adjusted to the correct phosphate concentration by adding equal molar amounts of K_2HPO_4 and KH_2PO_4. MOPS-MGS plates were solidified with 1.5% Difco Bacto Agar. Cultures were grown with 500 μg of streptomycin, 200 μg of neomycin, or 40 μg gentamicin per ml where appropriate and incubated for 96 h at 30°C.

Carbohydrate composition analyses. To characterize the sugar composition of the exopolysaccharides produced by *S. meliloti*, a culture was grown on a 0.1 or 100 mM MOPS-buffered minimal medium plate over a nylon filter disk, which was used to minimize carbohydrate transfer from the medium. The culture was suspended in 1 ml of distilled H_2O (dH_2O) and centrifuged at 20,800 × *g* for 3 min to pellet the cells. The supernatant was dialyzed against dH_2O for 2 days by using a 1000 MWCO membrane (Spectrum). The exopolysaccharides in the supernatant were hydrolyzed in 10 volumes of 2 M trifluoroacetic acid at 100°C for 18 h in a sealed glass vial. The hydrolyzed exopolysaccharides were dried in a Brinkmann SpeedVac centrifuge and resuspended in a small volume of water for analysis by high-pressure anion-exchange chromatography (HPAEC). HPAEC was performed on a Dionex DX 500 with a CarboPac MA1 (anion-exchange) column (4 by 250 mm) using a pulsed amperometric detector with a gold working electrode (Dionex). The waveform used for the analysis was as follows: 0.1 V for 0.4 s, 0.7 V for 0.2 s, and −0.1 V for 0.4 s. The samples were eluted isocratically by using 500 mM NaOH. The carbohydrate composition of the exopolysaccharides was calculated as a glucose/galactose ratio based on the elution times of known glucose and galactose standards. Because there was no free glucose or galactose before hydrolysis, all calculated Glc/Gal ratios represent hydrolyzed exopolysaccharide.

Quantitation of carbohydrate production. Quantitation of exopolysaccharide production by the various *S. meliloti* derivatives was performed with Dreywood's anthrone reagent (31). Cultures (2 ml) of MOPS-MGS medium with the appropriate phosphate concentrations were inoculated with the strains and grown for 72 h at 30°C. Cell density measurements were taken at an optical density of 600 nm (OD_{600}), and the cultures were centrifuged at 20,800 × *g* for 3 min to pellet the cells. The supernatants were tested for total carbohydrate content by the anthrone assay. Anthrone unit/OD_{600} ratios reflect exopolysaccharide production per cell density unit. Due to signal detection differences between glucose (1 U) and galactose (0.54 U) in the assay and the structures of the subunits of succinoglycan (7 Glc + 1 Gal = 7.54 U) and EPS II (1 Glc + 1 Gal = 1.54 U), a correction factor of 4.9 (7.54/1.54) was implemented for samples that only contain EPS II (*exoY* in 0 mM K_2HPO_4). Likewise, a correction was made for Rm1021 in 0 mM K_2HPO_4 because this strain synthesizes approximately equimolar amounts of succinoglycan and EPS II in low phosphate. Background anthrone-positive material in the cultures was determined by using an *exoY expA* strain, a derivative that produces neither exopolysaccharide.

Analysis of the molecular weight of EPS II. A 5-ml culture of a strain unable to produce succinoglycan (*exoY*) was grown in LB-MC for 48 h at 30°C. The culture was spun at 20,800 × *g* for 3 min to pellet the cells, washed twice with sterile dH_2O, and inoculated into 1 liter of MOPS-buffered minimal medium with 0 mM phosphate. The culture was incubated for 7 days in a rotary shaker and EPS II was isolated and assayed for molecular weight by HPAEC (18, 39). HPAEC was performed by using a P10 column matrix (Dionex), and samples were eluted in a gradient with 100 mM NaOH (eluent A) and 1 M sodium acetate in 100 mM NaOH (18, 39). The gradient was a linear variation of the proportion of eluent B, as follows: 0 min and 10% B, 3 min and 10% B, 10 min and 20% B, 25 min and 100% B, 50 min and 100% B, 55 min and 10% B, and 60 min and 10% B. The flow rate was 1 ml/min. The waveform used for the analysis was as follows: 0.05 V for 0.6 s, 0.75 V for 0.2 s, and −0.15 V for 0.4 s. EPS II purified from *expR101 exoY* (low- and high-molecular-weight EPS II) and *mucR exoY* (high-molecular-weight EPS II only) strains was used as a standard. These strains were grown and the EPS II was prepared and analyzed as previously described (18).

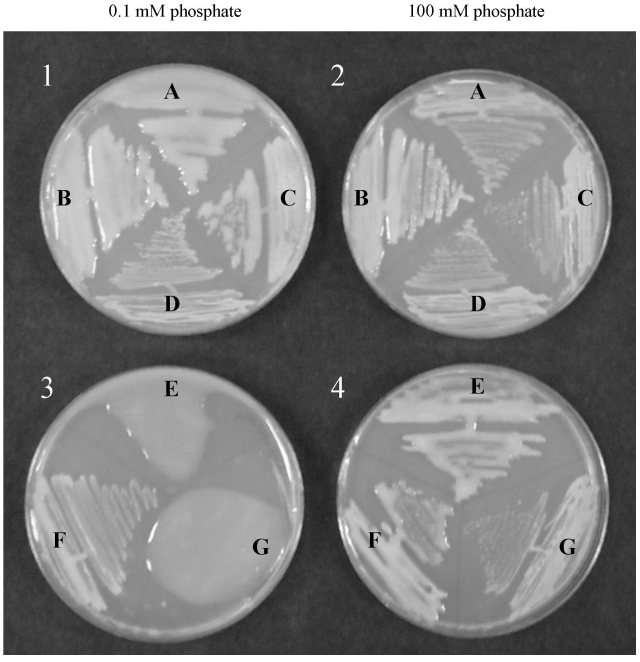

FIG. 1. Exopolysaccharide production by *S. meliloti* on plates with different phosphate concentrations. Plates with 0.1 mM (1 and 3) or 100 mM (2 and 4) K_2HPO_4 were prepared as indicated in Materials and Methods. Plates 1 and 2: A, Rm1021; B, *expA*; C, *exoY*; and D, *exoY expA*. Plates 3 and 4: E, *expR101*; F, *expR101 expA*; and G, *expR101 exoY*.

β-Galactosidase assay of gene expression. All cultures tested for β-galactosidase activity were grown to log phase in MOPS-MGS medium with different phosphate concentrations (0, 20, 100, 150, and 200 mM K_2HPO_4) at 30°C and assayed as previously described (30) to determine Miller units of activity.

Plant inoculation experiments. All indicated strains were incubated with *M. sativa* as previously described on standard (25) and modified Jensen's media. Modifications included omission of K_2HPO_4, exchange of $CaHPO_4$ with an equivalent amount of $CaCO_3$, and supplementation with 0.1 mM (low-phosphate) or 25 mM (high-phosphate) KH_2PO_4-K_2HPO_4 equimolar buffer as the sole phosphate source. Bacterial cultures were grown in 0 mM K_2HPO_4 MOPS-MGS (low), LB-MC (standard), or 150 mM K_2HPO_4 MGS (high) liquid medium for 72 h. Plants were scored for pink and white nodules after 30 days of growth at 25°C, 60% relative humidity, and a 16-h light cycle.

RESULTS

Low-phosphate conditions stimulate the synthesis of EPS II in *S. meliloti*. In order to stimulate the limiting phosphate concentrations that *Sinorhizobium* may encounter in its natural soil environment, MOPS-buffered minimal medium plates with 0.1 mM K_2HPO_4 (low phosphate) were prepared (44). Rm1021 (wild type) is very mucoid in these low-phosphate conditions (Fig. 1-1A). To determine which exopolysaccharides are produced by the wild-type strain Rm1021, strains with a mutation in a gene responsible for the production of each of the two known *S. meliloti* exopolysaccharides were tested in the same conditions. A strain with an *expA* mutation is unable to synthesize EPS II (15) but is mucoid under these conditions (Fig. 1-1B), suggesting that succinoglycan is a component of the exopolysaccharide produced by Rm1021. An *exoY* mutant cannot produce succinoglycan (25), but it also develops mucoid

TABLE 2. Carbohydrate composition analyses of exopolysaccharide production by *S. meliloti* derivatives

Strain	Glc/Gal ratio[a] at:	
	0.1 mM phosphate	100 mM phosphate
Rm1021	3.4:1	6.8:1
exoY	1.0:1	None
expA	7.2:1	6.2:1
exoY expA	None[b]	None
expR101	1.3:1	6.2:1
expR101 exoY	1.0:1	None
expR101 expA	7.6:1	6.4:1
phoB[c]	7.6:1	6.4:1
phoB exoY[c]	None	None
phoB expA[c]	7.6:1	6.2:1

[a] The ratios of glucose and galactose were calculated by total peak area. Glucose and galactose were assigned based on the chromatographic profiles of known carbohydrate standards.
[b] None, background levels of glucose due to cyclic glucan production.
[c] RmH615 is a derivative of Rm1021 with a *phoB* mutation.

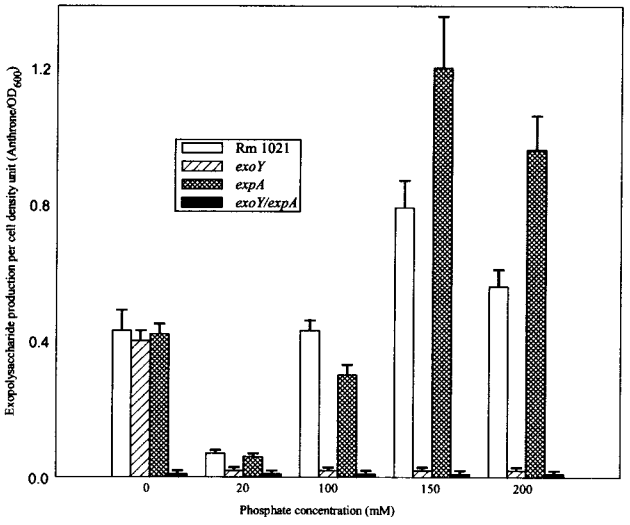

FIG. 2. Exopolysaccharide production by *S. meliloti* in different phosphate concentrations. Supernatant exopolysaccharides were purified as described in Materials and Methods, and the total carbohydrate content was determined by anthrone assay (31). The OD_{600} of each culture was determined, and the total carbohydrate per cell density unit was plotted against the concentration of phosphate in the growth medium.

colonies (Fig. 1-1C), indicating that EPS II also comprises part of the exopolysaccharide production in low phosphate. An *exoY expA* double mutant was constructed to establish the correlation between colony mucoidy and exopolysaccharide production. This strain is nonmucoid at all phosphate concentrations (Fig. 1-1D and 1-2D).

We also analyzed *expR101*, a Rm1021 derivative with a mutation that increases EPS II production (15), to determine if this strain behaved like Rm1021 in these different phosphate conditions. The *expR101* mutant and derivatives with either an *exoY* or *expA* mutation were plated on low-phosphate medium (Fig. 1-3). Because most of the mucoidy of the *expR101* strain is eliminated with the introduction of an *expA* mutation, it is apparent that the main component of the exopolysaccharides produced in low phosphate by the *expR101* mutant is EPS II. The *expR101 expA* derivative retained a low level of mucoidy, revealing that there is also some succinoglycan production at this low phosphate level.

To confirm biochemically which exopolysaccharides were produced by the various strains, we sampled the cultures grown on low-phosphate plates for carbohydrate composition analyses (Table 2). We took advantage of the very different glucose/galactose ratios of succinoglycan (7:1) (2) and EPS II (1:1) (21) to differentiate the two exopolysaccharides. Analysis of the exopolysaccharide production by the *exoY* derivative (1:1 Glc/Gal ratio) verifies that only EPS II is produced by this strain in low-phosphate conditions. Exopolysaccharide analysis of the *expA* derivative yields a ratio of 7.2:1, confirming that the exopolysaccharide produced by this strain in low phosphate is succinoglycan. The *exoY expA* double mutant yielded only background levels of glucose that we attribute to cyclic glucan production. Rm1021 produces exopolysaccharides with a Glc/Gal ratio of 3.4:1, suggesting that there is a mixture of succinoglycan and EPS II produced by wild-type *Sinorhizobium* in low phosphate concentrations. The results of the carbohydrate composition analyses for *expR101* (1.3:1 ratio) and *expR101 exoY* (1:1 ratio) strains support the observation that the exopolysaccharide produced by *expR101* in low phosphate is primarily EPS II. The Glc/Gal ratio of the *expR101 expA* strain (7.6:1) confirms that *expR101* derivatives retain the ability to produce succinoglycan in low phosphate levels.

A wild-type *S. meliloti* strain produces significant amounts of exopolysaccharides when grown at low levels of phosphate (0 mM), produces very little at intermediate levels of phosphate (20 mM), and achieves maximal production of exopolysaccharides in high-phosphate (150 mM) concentrations (Fig. 2). An *exoY* derivative of Rm1021 (which lacks the ability to synthesize succinoglycan) shows some exopolysaccharide production at low levels of phosphate concentration but produces negligible amounts of exopolysaccharide at higher concentrations of phosphate, confirming that most of the exopolysaccharide produced at high phosphate is indeed succinoglycan. On the other hand, an *expA* mutant (that is unable to synthesize EPS II) shows a pattern of exopolysaccharide production very similar to wild type throughout the entire phosphate concentration spectrum. Both Rm1021 and the *expA* mutant produce high levels of exopolysaccharides at higher levels of phosphate, although the absolute quantities of exopolysaccharides produced varied between the two strains. Interestingly, this mutant only produces succinoglycan, even at low concentrations of phosphate, suggesting that in an *exp* mutant background, succinoglycan production is derepressed at low phosphate concentrations. Based on this analysis, exopolysaccharide production under most laboratory conditions (LB-MC, MOPS-MGS, and Jensen's media) may be at minimal levels.

High-phosphate conditions block synthesis of EPS II and induce synthesis of succinoglycan. In order to observe the exopolysaccharide production of *S. meliloti* in the high-phosphate conditions present inside a plant nodule, MOPS-buffered minimal medium plates with 100 mM K_2HPO_4 (high phosphate) were prepared. Upon plating the strains on this medium, two dramatic effects were observed. First, no EPS II production could be detected in either Rm1021 or an *expR101* mutant in high-phosphate concentrations (Fig. 1-2C and 1-4G). An increase in phosphate concentration to 2 mM K_2HPO_4 was sufficient to block EPS II production in Rm1021 (reference 44 and data not shown), but an increase to 100 mM phosphate was required to block EPS II production in an *expR101* mutant (Fig. 1-4G). The *expR101 exoY* strain can only produce EPS II and is nonmucoid at 100 mM phosphate, suggesting that the EPS II production in an *expR101* back-

TABLE 3. *exo* and *exp* gene expression in different phosphate conditions in *S. meliloti*[a]

Strain and fusion	Mean Miller units ± SD of β-galactosidase activity at:				
	0 mM phosphate	20 mM phosphate	100 mM phosphate	150 mM phosphate	200 mM phosphate
Rm8501					
exoY-lacZ	8.1 ± 1.0	11 ± 0.7	37 ± 3.0	55 ± 4.0	120 ± 13
expA-lacZ	10 ± 1.5	1.9 ± 0.1	1.4 ± 0.2	1.7 ± 0.1	1.3 ± 0.5
RmH615					
exoY-lacZ	4.9 ± 1.0	8.5 ± 1.7	19 ± 2.0	98 ± 4.0	119 ± 13
expA-lacZ	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.7 ± 0.2

[a] Rm8501 is a *lac* mutant of Rm1021. RmH615 is a *lac* mutant and has a *phoB* mutation. All data are calculated in Miller units of β-galactosidase activity.

ground at this high phosphate concentration is very low or absent altogether.

We also observed that there was a large increase in the amount of mucoidy in succinoglycan-producing strains at high phosphate levels compared to the same strains grown on media with intermediate levels (2 to 20 mM K_2HPO_4) of phosphate (data not shown). Both Rm1021 and *expA* strains, which are capable of producing succinoglycan, became very mucoid at this level, while the *exoY* strain was nonmucoid (Fig. 1-2). Also, the mucoidy of *expR101* strain at this high phosphate concentration was eliminated with an *exoY* but not with an *expA* mutation (Fig. 1-4E, F, and G). This observation demonstrates that an *expR101* strain produces succinoglycan, not EPS II, at this high phosphate level.

We performed carbohydrate composition analyses to confirm which exopolysaccharides were produced at high phosphate levels. The Glc/Gal ratios of both wild-type Rm1021 (6.8:1) and *expA* (6.2:1) strains suggest that succinoglycan is the exopolysaccharide being produced under these high-phosphate conditions (Table 2). No EPS II production could be detected in either the *exoY* or the *expR101 exoY* strain in high phosphate. Analyses of *expR101* and *expR101 expA* strains yielded Glc/Gal ratios of 6.2:1 and 6.4:1, respectively. These ratios confirm that *expR101* strains, like Rm1021, produce succinoglycan at this high phosphate concentration. The dramatic change in the glucose/galactose ratio when an *expR101* mutant is grown in low (1.3:1 ratio)- and high (6.3:1 ratio)-phosphate conditions demonstrates how exopolysaccharide synthesis in this strain shifts from EPS II in low phosphate concentrations to succinoglycan in high-phosphate concentrations.

Exopolysaccharide gene expression is subject to phosphate-dependent regulation. We attempted to determine if there was a correlation between phosphate-dependent exopolysaccharide production and changes in exopolysaccharide gene expression. Chromosomal (single-copy) *exoY-lacZ* and *expA-lacZ* fusions were used to measure changes in expression from the genes responsible for the production of succinoglycan (*exo*) and EPS II (*exp*). In Rm8501 (a *lac* mutant of Rm1021), there is an eightfold decrease in transcription from the *exp* genes when the cells are grown in 200 mM (high) phosphate versus 0 mM (low) phosphate conditions (Table 3). In addition, most of this decrease occurs between 0 and 20 mM phosphate, suggesting that EPS II production in Rm1021 is only seen at very low levels of phosphate. This observation is consistent with the observed increase in EPS II synthesis by Rm1021 in low-phosphate conditions and is in agreement with the sevenfold increase in *exp* gene expression in low phosphate with an *exp-lacZ* fusion seen previously (40, 44). Conversely, expression from the *exo* genes increases 15-fold when the phosphate concentration in the medium is increased from 0 to 200 mM K_2HPO_4. This is consistent with the increase in exopolysaccharide production seen in succinoglycan-producing strains at high levels of phosphate. Thus, transcription from the *exp* genes decreases and transcription from the *exo* genes increases as phosphate levels rise. From these data, we suggest that one possible source of the differences in exopolysaccharide production at different levels of phosphate is the differential expression of the genes responsible for the production of succinoglycan and EPS II.

The EPS II produced in low-phosphate conditions by Rm1021 does not allow nodule invasion. It was previously shown that an active low-molecular-weight fraction of EPS II synthesized in strains with an *expR101* mutation could function in lieu of succinoglycan in nodule invasion (15). We investigated how exopolysaccharide production in different phosphate concentrations, especially EPS II production by Rm1021, might affect the symbiosis between *Sinorhizobium* and *M. sativa*. Using modified Jensen's agar, the exopolysaccharide mutants were incubated with *M. sativa* on high-, standard-, and low-phosphate plant media. Standard Jensen's medium (25) has a total phosphate concentration of 8.5 mM, an intermediate concentration between the low (0.1 mM)- and high (25 mM)-phosphate Jensen's media. Attempts to create Jensen's agar with higher phosphate concentrations (50 or 100 mM) resulted in wilting and death of the plants due to high salt concentrations. The Rm1021, *expA*, and *expR101* strains are capable of producing succinoglycan and effectively invaded nodules at all three levels of phosphate. Interestingly, the invasion efficiency of the *expR101 exoY* strain was poor on high-phosphate medium (Table 4). This is consistent with the observation that EPS II production is very low at this level, even in a strain with an *expR101* mutation. We also observed that the *exoY* mutant did not invade nodules in any phosphate conditions, even though there is increased EPS II production

TABLE 4. Nodule invasion phenotype of *S. meliloti* exopolysaccharide mutants on modified Jensen's media

Strain	No. of plants with pink nodules[a] at:		
	0.1 mM phosphate	8.5 mM phosphate	25 mM phosphate
Rm1021	30	30	30
exoY	0	0	0
expA	30	30	30
exoY expA	0	0	0
expR101	30	30	30
expR101 exoY	30	30	10[b]

[a] Plants without pink nodules developed white nodules characteristic of inoculation of *M. sativa* with an *exo* mutant.

[b] The plants had an average of 1.6 pink nodules per plant versus 9.1 pink nodules per plant for those inoculated with *expR101* in high-phosphate media.

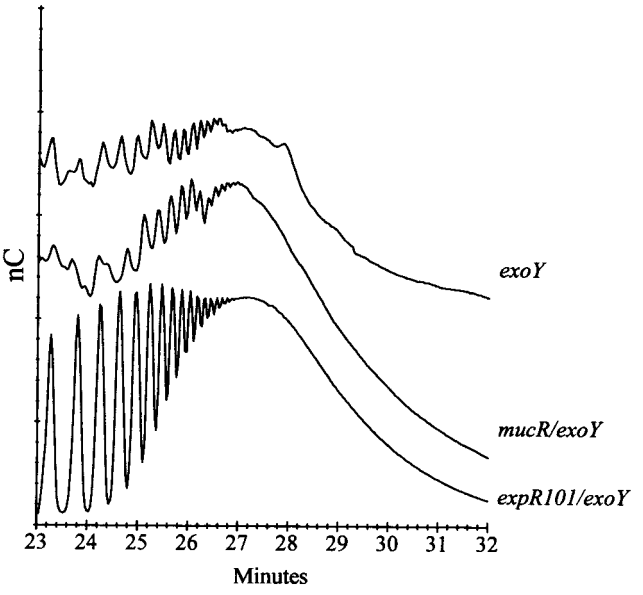

FIG. 3. Chromatographic analysis of the molecular weight of EPS II produced by an *exoY* strain in low phosphate. The graph is a vertical overlay of chromatographic profiles of three independent samples. Separation by HPAEC of EPS II was performed as previously described (18). The carbohydrate electrochemical response is expressed in nanocoulombs. Purified EPS II from *mucR exoY* and *expR101 exoY* strains was used as a standard (18). The biologically active fraction of EPS II elutes with a retention time of 22 to 26 min.

in this strain at low phosphate levels. The *expR101 exoY* strain, however, did effectively invade nodules in the same low-phosphate conditions, even though it exclusively produced EPS II as well. These results suggested that there could be a difference in the EPS II being produced by the *exoY* and *expR101 exoY* strains.

The EPS II induced in low-phosphate conditions lacks the low-molecular-weight fraction crucial for efficient nodule invasion. Because the EPS II produced by the *exoY* derivative in low phosphate did not allow nodule invasion, we analyzed whether the EPS II contained the molecular weight fraction that is symbiotically active in nodule invasion (18). Two strains with mutations that increase EPS II production in Rm1021 were used as controls. The MucR protein is a repressor of EPS II production in *S. meliloti* (34), but the EPS II produced by a *mucR* derivative does not allow nodule invasion (33). Analyses have revealed that the EPS II produced by *mucR* derivatives is composed primarily of high-molecular-weight chains and lacks the active fraction (15 to 20 dimer subunits) found in strains with the *expR101* mutation (18). To determine how the molecular weight of EPS II from an *exoY* derivative compared to that produced by *expR101 exoY* and *mucR exoY* strains, EPS II from a culture of each strain was purified and analyzed by HPAEC (Fig. 3). The *expR101 exoY* mutant produces a large amount of the biologically active fraction of low-molecular-weight EPS II that elutes with a retention time of 22 to 26 min and high-molecular-weight EPS II that elutes between 26 and 30 min (18). The EPS II from a *mucR exoY* mutant, however, is mostly high molecular weight. The EPS II from an *exoY* mutant grown in low-phosphate medium also lacks the low-molecular-weight EPS II fraction that is active in nodule invasion. The EPS II isolated from both *exoY* and *mucR exoY* is composed of primarily high-molecular-weight polymers.

A functional *phoB* gene is required for production of EPS II at low phosphate concentrations. We were interested in determining if this pattern of phosphate-dependent exopolysaccharide production in *S. meliloti* could be subject to PhoB-dependent regulation. It has been shown that some genes that are not directly involved in the acquisition and assimilation of phosphate are regulated by the PhoB protein in *S. meliloti* (42). The PhoB protein is a transcriptional regulator that binds to characteristic sequences (called *pho* boxes) upstream from phosphate-regulated genes during phosphate starvation (29). Binding of PhoB to these sequences can increase (4) or decrease (6, 41) transcription of these genes, depending on the location of the *pho* boxes in the promoter region. Previous work by Zhan et al. (44) demonstrated that Rm8002, a derivative of Rm1021 with an uncharacterized mutation that affected alkaline phosphatase activity, failed to induce EPS II production in low-phosphate conditions. Later, Summers et al. found the *expC* gene (a gene in the *exp* operon responsible for EPS II production) during a search for genes that were expressed differently in low phosphate (42). They demonstrated that this difference was eliminated with a *phoB* mutation. RmH615 is a derivative of Rm1021 that has a *phoB* mutation (4). In high-phosphate conditions, RmH615 behaves much like Rm1021. The mucoidy of RmH615 colonies is eliminated with the introduction of an *exoY* mutation, and carbohydrate analyses of the exopolysaccharides produced by RmH615 (a 6.3:1 Glc/Gal ratio) confirm that succinoglycan is the exopolysaccharide produced at high phosphate (Table 2). When RmH615 (*phoB*) is grown in low-phosphate conditions, however, the mucoidy that results is eliminated by the introduction of an *exoY* mutation into the strain but is not affected by an *expA* mutation. Unlike Rm1021 or the *expR101* mutant, there appears to be no EPS II production in low phosphate in RmH615. Carbohydrate composition analyses confirm that succinoglycan is the only exopolysaccharide produced in low phosphate (a 7.6:1 Glc/Gal ratio), and only background levels of glucose were detected in the RmH615 *exoY* double mutant (Table 2). Analysis of the RmH615 *expA* double mutant revealed a Glc/Gal ratio that was identical (i.e., 7.6:1) to that for RmH615, indicating that an *expA* mutation does not affect exopolysaccharide production in this strain. While β-galactosidase assays in RmH615 resulted in a pattern of *exo* gene expression that was similar to Rm1021, only background levels of *exp* expression were detected at all phosphate levels (Table 3).

DISCUSSION

In this report we have analyzed how phosphate concentration has a regulatory effect on exopolysaccharide production. In Luria broth, Rm1021 synthesizes low levels of succinoglycan and very little (if any) EPS II (15, 34). Jensen's agar, the medium used to determine nodulation efficiency, contains 8.5 mM total phosphate, which is between 10^3- and 10^4-fold higher than the natural soil phosphate concentration. The large difference in phosphate concentrations between the soil and the nodule is an example of the dissimilar conditions that *Sinorhizobium* may encounter during its lifetime, and certain physiological adjustments may occur to satisfy the requirements of these environments. Exopolysaccharides may play a role in the transition between the soil and the plant environments. In both Rm1021 and the *expR101* strain, increasing the phosphate concentration decreases EPS II production to very low levels. Merely increasing the osmolarity of the medium has been previously shown (10) to have no significant effect on either the amount of succinoglycan produced or the expression of the *exo* genes. EPS II production cannot be detected in Rm1021 when the phosphate level is raised to 2 mM and in the *expR101* background when the phosphate level is raised to 100 mM.

Because EPS II production is greatest at low phosphate levels and the nodule is a high-phosphate environment, we assume that EPS II is produced primarily while *Sinorhizobium* is in the soil and not inside the nodule. This low level of EPS II production in high-phosphate conditions is evident since the *expR101 exoY* strain is very inefficient in invading alfalfa nodules on high-phosphate Jensen's media. The *expR101 exoY* mutant is invasion proficient on low- and standard-phosphate Jensen's media, but most of the plants inoculated with this strain on the high-phosphate medium failed to develop any pink nodules. In this example, the invasion phenotype of a strain depends on the phosphate concentration of the medium.

In general, *S. meliloti* strains that exclusively produce EPS II invade *M. sativa* with a much lower efficiency than do succinoglycan-producing strains (15) (Table 4). While EPS II produced in an *expR101* background is functional in nodule invasion, the lack of pink nodules on plants inoculated with an *exoY* mutant in low-phosphate conditions suggests that the EPS II produced by Rm1021 in low-phosphate conditions does not allow invasion of nodules. The molecular weight of exopolysaccharides in *Sinorhizobium* is critical in allowing efficient infection of nodules (7, 18–20, 43). Both succinoglycan and EPS II are known to have low-molecular-weight fractions that are essential for nodule invasion. Addition of succinoglycan trimer in *trans* rescues the nodule invasion defects of an *exo* mutant (7, 43). A fraction of 15 to 20 dimer subunits of EPS II in concentrations that are as low as 7 pmol also allows invasion of nodules by *exo* derivatives (18). We show that the EPS II produced by *exoY* lacks the low-molecular-weight fraction shown to be symbiotically active in nodule invasion. The EPS II from the *exoY* derivative closely resembles the high-molecular-weight EPS II produced by a *mucR exoY* derivative, which does not invade nodules despite overproducing EPS II (18) (Fig. 3).

The observation that EPS II from the *exoY* strain does not allow nodule invasion leads to the question of what role EPS II may play in *Sinorhizobium*-alfalfa symbiosis. Our data suggest that the lack of EPS II production by *expA* does not affect the efficiency of nodule invasion in the conditions we use to assay invasion. Because production of EPS II in the amounts we have observed at low phosphate is energetically expensive, it probably has a role important to the bacteria. Perhaps EPS II is important to *S. meliloti* as a free-living organism. For example, EPS II could mitigate the effects of desiccation or neutralize potentially harmful compounds in the soil. On the other hand, EPS II production in low phosphate could increase the host specificity of *S. meliloti* for *M. sativa*. It has been previously shown that EPS II does not substitute for succinoglycan in nodule invasion on several hosts that can be effectively nodulated by Rm1021 derivatives that produce succinoglycan (15).

High-phosphate conditions, such as those probably found inside the plant nodule, inhibit EPS II production and stimulate succinoglycan production. Analyses of Rm1021 and *expR101* in high-phosphate conditions (Fig. 1 and 2) suggest that succinoglycan appears to be the primary exopolysaccharide produced at these levels. The fact that *expR101* produces large amounts of succinoglycan in high-phosphate conditions is novel, since *expR101* strains have been observed to produce mostly EPS II and suppress succinoglycan production in *S. meliloti* under most experimental conditions. The function of high levels of succinoglycan production in high-phosphate conditions is not yet clear. β-Galactosidase assays suggest a 15-fold increase in transcription from the *exo* genes in high-phosphate conditions (Table 2). In Rm1021, succinoglycan production is essential for nodule invasion (*exoY* derivatives do not invade nodules in any phosphate conditions), but succinoglycan may also have a function inside the nodule. Succinoglycan production in the nodule could protect *Sinorhizobium* from plant defense mechanisms or other adverse conditions inside the nodule.

The details of how exopolysaccharide production is regulated by phosphate remain to be elucidated. We show that no EPS II production occurs in a *phoB* null strain in low-phosphate conditions. It has been demonstrated in *Escherichia coli* that the PhoB protein binds to its target DNA sequence in conditions of phosphate starvation (29). Upon binding, PhoB can either increase or decrease transcription from the genes that it regulates, depending on the position of the *pho* box with respect to the RNA polymerase binding site. In high-phosphate conditions, the PhoB protein becomes phosphorylated and is released from the *pho* box (29). Unlike Rm1021, there is no EPS II production induced at low phosphate in a *phoB* mutant. Carbohydrate composition analysis confirms that succinoglycan (a 7.6:1 Glc/Gal ratio) is the sole exopolysaccharide produced by a *phoB* mutant, and β-galactosidase assays show no stimulation in transcription from the *exp* genes in low phosphate (40, 42). This observation is in agreement with the differential expression of the *expC* gene in low phosphate observed by Summers et al. (42). These observations suggest that PhoB positively regulates the expression of the *exp* genes, but whether this control is direct or indirect has yet to be resolved.

A possibility of indirect regulation of the *exp* genes by PhoB is through regulation of phosphate transport. The phosphate transport systems of *S. meliloti* have been elegantly characterized by others (1, 4, 5, 32). The *phoCDET* operon encodes a phosphate transport system that is induced in limiting phosphate conditions but repressed under high-phosphate conditions (4). The *orfA-pit* operon encodes a phosphate transport system that is activated when *S. meliloti* is grown in excess phosphate but repressed in limiting phosphate conditions (6). The common regulator for these two systems is the PhoB protein. The effect of a *phoB* mutant on phosphate transport is dual. Transcription from the *phoCDET* phosphate transport system is suppressed and transcription from the *orfA-pit* operon is stimulated (6). Perhaps *phoCDET* transcription, which is suppressed in a *phoB* background, is used as a signal of low-phosphate conditions and is required for EPS II production in low phosphate. We are currently investigating how the products of the *phoCDET* and *orfA-pit* operons affect exopolysaccharide production in *S. meliloti*.

Previous work has suggested that the *exp* operon may have sites that are responsible for the induction of EPS II production in low-phosphate conditions. Mutation of the *mucS* locus of the *exp* operon eliminates the induction of EPS II synthesis in low phosphate (3). Due to the position of the *mucS* locus in the intergenic region between the two oppositely transcribed arms of the *exp* operon, this site could act as a regulatory region for the operon. A study by Summers et al. has suggested a region of limited homology to a *pho* box within the *mucS* locus (42), and others have suggested regions of weak homology throughout the *exp* operon (40). We have noticed a very strong putative *pho* box about 150 bp upstream of this locus, but the significance of this region is still unknown.

We also noticed the presence of two putative PhoB-binding elements upstream from the promoter region of *exoY*. Due to its position between the two oppositely transcribed arms of the *exo* operon, the *exoX-exoY* intergenic region has been suggested to be a logical site for transcriptional regulation of the *exo* operon. Recent work has demonstrated that the *exoX-exoY* intergenic region may have sites for binding of the MucR protein, a positive regulator of succinoglycan production (10).

The first of the two putative *pho* boxes resides upstream (−63 to −46) of a putative start site for *exoY* and is very similar in placement to a putative *pho* box upstream (−62 to −45) of the phosphate-regulated *phoCDET* operon in *S. meliloti* (4). The second box, however, has an unusual placement (−27 to −10 versus −40 to −23 in *phoCDET*) that could block the ribosome-binding site when PhoB binds in low-phosphate conditions. We do not know what role, if any, these putative PhoB-binding regions may have on the production of succinoglycan and/or expression of the *exo* genes. Site-directed mutagenesis of these putative PhoB-binding elements could elucidate what role, if any, PhoB could play in succinoglycan production. The environment that *Sinorhizobium* encounters as a free-living organism in the soil is very different from that found inside the nodule. The radical change in phosphate concentration between the soil and the nodule could provide a strong signal of the changing environmental conditions. Many other environmental signals may play a role in facilitating the adjustment of the bacteria between the soil and the nodule. It would be interesting to see how other factors important to symbiosis may be affected by changes in phosphate concentration and what other signals may play a role in controlling exopolysaccharide production in *Sinorhizobium*.

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