JOURNAL OF BACTERIOLOGY, Nov. 1991, p. 7391–7394
0021-9193/91/227391-04$02.00/0

Vol. 173, No. 22

Induction of the Second Exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by Low Phosphate Concentrations

HANGJUN ZHAN, CHI CHANG LEE, AND JOHN A. LEIGH*

Department of Microbiology SC-42, University of Washington, Seattle, Washington 98195

Received 27 June 1991/Accepted 5 September 1991

In previous work, *Rhizobium meliloti* SU47 produced its alternative exopolysaccharide (EPSb [also called EPS II]) only in strains that were genetically altered to activate EPSb synthesis. Here we report that EPSb synthesis is not entirely cryptic but occurred under conditions of limiting phosphate. This was shown in several different *exo* mutants that are blocked in the synthesis of the normal exopolysaccharide, succinoglycan. In addition, EPSb biosynthetic gene expression was markedly increased by limiting phosphate. An apparent regulatory mutant that does not express alkaline phosphatase activity was unable to produce EPSb under these conditions. A *mucR* mutant that was previously shown to produce EPSb instead of the normal exopolysaccharide, succinoglycan, was not sensitive to phosphate inhibition of EPSb synthesis. No evidence was found to indicate that *exoX*, which affects succinoglycan synthesis, had any influence on EPSb synthesis. In contrast to limiting phosphate, limiting nitrogen or sulfur did not stimulate EPSb synthesis as it does succinoglycan.

Several environmental stresses tend to stimulate bacterial exopolysaccharide (EPS) production. Limiting for nitrogen, phosphorus, or sulfur stimulates EPS synthesis in a variety of organisms (20). It has been shown that in *Pseudomonas aeruginosa*, starvation for nitrogen or phosphate, or increased medium osmolarity, activates the expression of *algD*, an alginate synthesis gene (1, 3). It has also been demonstrated that prolonged exposure to dehydrating agents allows *P. aeruginosa* to switch from a nonmucoid to a mucoid or alginate-producing form (4).

The EPS that *Rhizobium meliloti* SU47 normally produces is succinoglycan. However, a second EPS (EPSb [also called EPS II]) is produced in strains containing mutations in *mucR* (26) or *expR* (9) and in strains containing extra copies of a gene cluster involved in EPSb synthesis (9, 26). EPSb differs markedly in structure from succinoglycan (13), and genetic studies indicate that EPSb synthesis is independent of succinoglycan synthesis (9, 26). EPSb can functionally replace succinoglycan in alfalfa root nodule invasion but is not required for invasion in a succinoglycan-producing background (9, 26).

An unusual feature of EPSb was that it appeared to be cryptic. EPSb production was not observed with wild-type *R. meliloti* or with *exo* mutants, which are blocked in succinoglycan production (11, 12, 15). This was the case even under conditions such as limiting nitrogen that favored succinoglycan synthesis. Nor did EPSb appear to be synthesized during nodulation, since *exo* mutants did not invade. Here we report that under a relatively restricted set of environmental conditions, EPSb is produced by *R. meliloti* *exo* mutants.

To study the effect of limiting phosphate, we used a modified morpholinopropane sulfonate (MOPS)-buffered medium (18) containing 50 mM potassium-MOPS (pH 7.4), 55 mM mannitol, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 19 mM sodium glutamate, and 0.004 mM biotin. In some cases, 19 mM NH_4Cl was used in place of sodium glutamate. Either 0.1 mM (low-phosphate) or 2.0 mM (high-phosphate) K_2HPO_4 was added. The effects of nitrogen and sulfur were tested by using M9 (16) medium containing 1% mannitol as a carbon source and various nitrogen sources in place of NH_4Cl or $MgCl_2$ in place of $MgSO_4$. The effect of osmolarity was tested by using GYM (7) medium in the presence or absence of 28 mM mannitol or 200 mM NaCl. The effect of desiccation was tested by adding 1 or 3% ethanol to YM (22), LB (16), or M9 medium as previously described (4). Unless otherwise stated, all experiments were done with medium solidified with 1.5% Difco Bacto agar. Cultures were incubated at 30°C and scored for mucoidy. Staining with Calcofluor, an indication of the presence of succinoglycan, was tested at the end of the incubation period by smearing growth onto Calcofluor-containing agar medium (12) and observing fluorescence after 10 min. Proton nuclear magnetic resonance spectroscopy, carbohydrate assay by the anthrone-sulfuric acid method, and column chromatography were performed as before (11). β-Galactosidase activity (17) and alkaline phosphatase activity (2) were measured as described previously. Bacterial strains used in this study are listed in Table 1. The *mucR* mutants were made by transducing (8) the Tn*5* markers from Rm5000 into Rm1021. Double mutants were made by transduction of the appropriate transposon markers as described previously (5, 8).

Low-phosphate stimulation of EPSb synthesis and EPSb-related gene expression. With 0.1 mM phosphate but not with 2.0 mM phosphate, colonies of *R. meliloti* appeared mucoid after 3 days (Table 2). In the case of the wild-type strain, Rm1021, this reflects the previously reported stimulation of succinoglycan synthesis by low phosphate concentrations (12). More importantly, the same stimulation was observed with *exo* mutants that are deficient in succinoglycan synthesis (Table 2 and Fig. 1). The same result was obtained with either 19 mM NH_4Cl or 19 mM sodium glutamate as the nitrogen source. In contrast, *exoB* and *exoC* mutants, which are deficient in both succinoglycan and EPSb production (9, 26), were nonmucoid, as was a double *mucA exoF* mutant specifically blocked in both succinoglycan and EPSb synthesis (26). (The control strain, Rm1021 *mucA*, is blocked in EPSb synthesis and produced succinoglycan [26].) These observations, together with the lack of Calcofluor staining in the appropriate cases, suggested that EPSb was being produced in response to low phosphate concentrations. With 2.0

* Corresponding author.

TABLE 1. Bacterial strains

R. meliloti strain or plasmid	Relevant characteristic(s)	Source or reference
Strain		
SU47	Wild type	21
Rm1021	SU47 Sm^r	F. Ausubel
Rm7061	Rm1021 *exoA61*::Tn*5*	12
Rm6087	Rm1021 *exoF55*::Tn*5*-233	5
Rm8332	Rm1021 *exoQ332*::Tn*5*	15
Rm1021ΔHKm	Rm1021 *exo* deletion, Nm^r	10
Rm7094	Rm1021 *exoB94*::Tn*5*	12
Rm7025	Rm1021 *exoC15*::Tn*5*	12
Rm7201	Rm1021 *mucA201*::Tn*5*	26
Rm8002	Rm1021 Pho^-	14
Rm7103	Rm1021 *exoX103*::Tn*5*	25
Rm7095	Rm1021 *exoR95*::Tn*5*	6
Rm7096	Rm1021 *exoS96*::Tn*5*	6
Rm7210	Rm1021 *mucR12*::Tn*5*	This work
Rm7211	Rm1021 *mucR12*::Tn*5*-233	This work
Rm5000	SU47 Rif^r	T. Finan
Rm7012	Rm5000 *mucR12*::Tn*5*	26
Rm7010	Rm5000 *mucR12*::Tn*5*-233	26
Plasmid pMuc	pLAFR1 cosmid clone of EPSb synthesis genes	26

mM phosphate, *exoA*, *exoQ*, and Rm1021ΔHKm remained nonmucoid even after 10 days of incubation.

To confirm the nature of the EPS produced under low-phosphate conditions, we obtained a water suspension of *exoQ* grown on MOPS agar medium with 0.1 mM phosphate for 6 days, centrifuged the suspension to remove cells, and performed proton nuclear magnetic resonance spectroscopy. The spectrum (Fig. 2) confirmed that the EPS was EPSb. We also examined the EPS produced by *exoQ* in liquid MOPS–0.1 mM phosphate medium. After 6 days of incubation, 0.74 mg of glucose equivalents per ml were produced, as opposed to 0.04 mg/ml in the presence of 1.0 mM K_2HPO_4. The carbohydrate was fractionated by Biogel A5m chromatography (11) yielding mainly high-molecular-weight carbohydrate that eluted with the void volume. This material consisted of EPSb as determined by proton nuclear magnetic cresonance spectroscopy (data not shown).

Phosphate limitation also induced the expression of a gene that was required for EPSb production. We constructed a *lacZ* fusion in a cluster of genes required for EPSb synthesis (9, 26) by the introduction of Tn*3*HoKm into the cosmid pMuc followed by recombination into the genome as described previously (9). That the fusion insertion, *muc-13*::Tn*3*HoKm, lay within a gene required for EPSb synthesis was shown by the observation that Rm1021 *mucR*::Tn*5*-233 *muc-13*::Tn*3*HoKm failed to produce EPSb. In 3-day liquid cultures, Rm1021 *muc-13*::Tn*3*HoKm produced 57 U of β-galactosidase activity in MOPS–0.1 mM phosphate and only 8.0 U in 2.0 mM phosphate. Background in Rm1021 was 2.3 and 2.4 U, respectively. This result indicated that at least one effect of low phosphate concentration on EPSb synthesis is at the level of gene expression.

TABLE 2. Mucoidy and Calcofluor staining of *R. meliloti* strains on MOPS agar medium with low and high phosphate concentrations

Strain	Presence of mucoidy (Calcofluor staining) with phosphate concn (mM) of[a]:	
	0.1	2.0
Rm1021 (wild type)	++ (+)	+/−
Rm1021 *exoA*, *exoF*, *exoQ*, or Δ[b]	++ (−)	−
Rm1021 *exoB* or *exoC*	− (−)	−
Rm1021 *mucA*	++ (+)	−
Rm1021 *mucA exoF*	− (−)	−
Rm8002 *exoA* or *exoQ*	−	−
Rm8002 (Pho^-)	++ (+)	−
Rm1021 *mucR*	+++ (−)	+++
Rm1021 *mucR exoA*	+++ (−)	+++
Rm8002 *mucR*	+++ (−)	ND[c]
Rm1021 *exoX*	+++ (+)	+++
Rm1021 *exoX exoF*	++ (−)	−

[a] Symbols indicate the degree of mucoidy, while symbols in parentheses indicate the presence (+) or absence (−) of succinoglycan as determined by Calcofluor staining. +++, strongly positive; ++, positive; +/−, equivocal; −, negative.

[b] Δ, Rm1021ΔHKm, a strain that carries a deletion of a major portion of the *exo* region (10).

[c] ND, not determined.

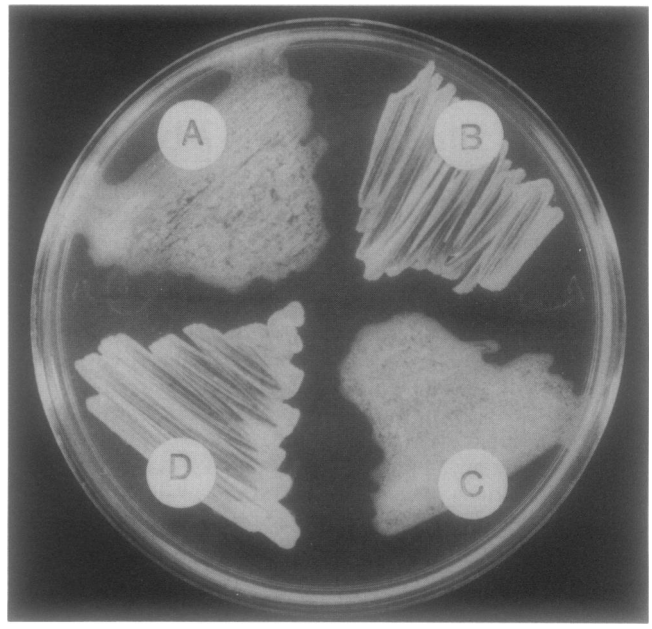

FIG. 1. Mucoidy of *R. meliloti* strains on MOPS–0.1 mM phosphate agar. A, Rm1021 *exoQ*; B, Rm1021 *mucA exoF*; C, Rm1021 ΔHKm; and D, Rm1021 *exoB*. Incubation time, 8 days.

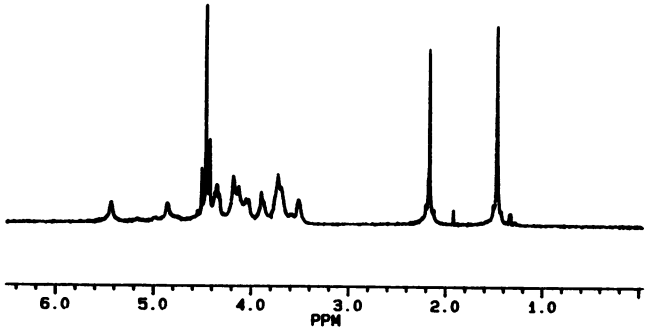

FIG. 2. Proton nuclear magnetic resonance spectrum of EPS from Rm1021 *exoQ*, showing similarity to EPSb (26).

TABLE 3. Mucoidy and Calcofluor staining of *R. meliloti* strains on M9 agar with various nitrogen sources

Strain	Presence of mucoidy (Calcofluor staining) with nitrogen source[a]				
	Glu (2 g/liter)	NO_3^- (2 g/liter)	Glu + NO_3^-[b]	NH_4^+ (0.05 g/liter)	NH_4^+ (2 g/liter)
Rm1021 (wild type)	+ (+)	+ (+)	+ (+)	+ (+)	− (+)
Rm1021 *exoA*, *exoF*, *exoQ*, or Δ[c]	− (−)	− (−)	− (−)	− (−)	− (−)

[a] Glu, sodium glutamate; NO_3^-, $NaNO_3$; NH_4^+, NH_4Cl.
[b] Glu and NO_3^- each at 2 g/liter.
[c] Δ, Rm1021ΔHKm.

Common control of alkaline phosphatase and EPSb synthesis. We wanted to test whether EPSb stimulation by low phosphate concentrations might involve a general phosphate starvation response. In *Escherichia coli*, limiting phosphate induces alkaline phosphatase activity (24). In *R. meliloti*, the same low-phosphate conditions that stimulated EPSb synthesis also stimulated alkaline phosphatase activity. Thus, an overnight culture of Rm1021 in LB Ca^{2+} Mg^{2+} (12) was obtained, and the cells were pelleted, washed three times with H_2O, and inoculated into MOPS liquid media containing 0.1 mM and 2.0 mM phosphate. After 8 h of incubation, alkaline phosphatase activity in 0.1 mM phosphate was 108.3 U but in 2.0 mM phosphate was only 4.8 U.

The response of EPSb synthesis to low phosphate concentrations was eliminated by a mutation that also appeared to affect a general phosphate starvation response. Rm8002 is an ethyl methanesulfonate-generated mutant of Rm1021 that was selected for the loss of alkaline phosphatase activity (14). This mutant expressed less than 0.1 U of alkaline phosphatase in both low- and high-phosphate media. The mutation appears to disrupt a regulatory gene, such as the *E. coli phoB* gene (24), since Rm8002 was deficient in the utilization of several different organic phosphate sources (23). *exo* mutants in the Rm8002 background were nonmucoid on MOPS–0.1 mM phosphate medium (Table 2), indicating that EPSb synthesis did not occur in this background. This was not due to an inherent inability of Rm8002 to produce EPSb, since Rm8002 *mucR*::Tn*5* was mucoid (Table 2; also, see below). In contrast, succinoglycan synthesis continued to respond to low phosphate concentrations in Rm8002 (Table 2).

Effects of known EPS regulatory mutations on EPSb synthesis. Phosphate did not affect mucoidy in a *mucR* mutant that was previously shown to produce EPSb rather than succinoglycan (26) or in a *mucR exoA* double mutant (Table 2). When the development of mucoidy was monitored with time, no difference between 0.1 mM and 2.0 mM phosphate could be discerned. This result indicates that the *mucR* mutation either alters the mechanism that mediates the response to phosphate or bypasses phosphate regulation by affecting another regulatory mechanism. The *mucR* mutation did not restore alkaline phosphatase activity in a Rm8002 background.

exoX, a mutation that increases succinoglycan production (19, 25), had no apparent effect on EPSb synthesis (Table 2), in agreement with an earlier report (19). The effects of *exoR* and *exoS* mutations (6) on EPSb synthesis were not tested, since double mutants (*exoR exoF* and *exoS exoF*) did not grow on MOPS medium or YM medium.

Although nitrogen limitation stimulated succinoglycan synthesis as shown before (6, 12), it did not induce EPSb synthesis (Table 3). Other conditions that did not induce mucoidy were low sulfur, dehydration, and high osmolarity (not shown).

In *R. meliloti*, the stimulation of succinoglycan synthesis by limiting nitrogen, phosphate, or sulfur has been reported (6, 12). In contrast, we have shown here that the stimulation of EPSb synthesis is restricted to phosphate limitation. This response appears to be regulated by a phosphate-sensitive system that also affects alkaline phosphatase expression. As in *P. aeruginosa* (3), the phosphate effect was also manifested at the level of gene expression. A regulatory mutation (*mucR*) previously shown to stimulate EPSb synthesis altered the response of EPSb to phosphate, while a mutation (*exoX*) that increased succinoglycan synthesis appeared to be restricted in its effect to that EPS. Further studies of the *mucR* gene and the genes involved in phosphate regulation will shed light on the mechanism of EPSb regulation.

We thank Graham Walker for Rm8002.
This work was supported by Public Health Service grant GM39785 from the National Institutes of Health.

REFERENCES

1. **Berry, A., J. D. De Vault, and A. M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmuoid *Pseudomonas aeruginosa strains*. J. Bacteriol **171**:2312–2317.
2. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and φ80 transducing lysates. J. Mol. Biol. **96**:307–316.
3. **De Vault, J. D., A. Berry, T. K. Misra, A. Darzins, and A. M. Chakrabarty.** 1989. Environmental sensory signals and microbial pathogenesis: *Pseudomonas aeruginosa* infection in cystic fibrosis. Bio/Technology **5**:352–357.
4. **De Vault, J. D., K. Kimbara, and A. M. Chakrabarty.** 1990. Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates alginate gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. Mol. Microbiol. **4**:737–745.
5. **De Vos, G. F., G. C. Walker, and E. R. Signer.** 1986. Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn*5* insertions. Mol. Gen. Genet. **204**:485–491.
6. **Doherty, D., J. A. Leigh, J. Glazebrook, and G. C. Walker.** 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic Calcofluor-binding exopolysaccharide. J. Bacteriol. **170**: 4249–4256.
7. **Dylan, T., D. R. Helinski, and G. S. Ditta.** 1990. Hypoosmotic adaptation in *Rhizobium meliloti* requires β-(1→2)-glucan. J. Bacteriol. **172**:1400–1408.
8. **Finan, T. M., E. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120–124.
9. **Glazebrook, J., and G. C. Walker.** 1989. A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56**:661–672.
10. **Gray, J. X., H. Zhan, S. B. Levery, L. Battisti, B. G. Rolfe, and J. A. Leigh.** 1991. Heterologous exopolysaccharide production in *Rhizobium* sp. strain NGR234 and consequences for nodule development. J. Bacteriol. **173**:3066–3077.
11. **Leigh, J. A., and C. C. Lee.** 1988. Characterization of polysac-

charides of *Rhizobium meliloti exo* mutants that form ineffective nodules. J. Bacteriol. **170**:3327–3332.
12. **Leigh, J. A., E. R. Signer, and G. C. Walker.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82**:6231–6235.
13. **Levery, S. B., H. Zhan, C. C. Lee, J. A. Leigh, and S. Hakomori.** 1991. Structural analysis of a second acidic exopolysaccharide of *Rhizobium meliloti* that can function in alfalfa root nodule invasion. Carbohydr. Res. **210**:339–347.
14. **Long, S., S. McCune, and G. C. Walker.** 1988. Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. J. Bacteriol. **170**:4257–4265.
15. **Long, S., J. W. Reed, J. Himawan, and G. C. Walker.** 1988. Genetic analysis of a cluster of genes required for the synthesis of the Calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J. Bacteriol. **170**:4239–4248.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119**:736–747.
19. **Reed, J. W., M. Capage, and G. C. Walker.** 1991. *Rhizobium meliloti exoG* and *exoJ* mutations affect the ExoX-ExoY system for modulation of exopolysaccharide production. J. Bacteriol. **173**:3776–3788.
20. **Sutherland, I. W.** 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23**:79–150.
21. **Vincent, J. M.** 1941. Serological properties of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. Proc. Linn. Soc. N. S. W. **66**:145–154.
22. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria, p. 3. International biology programme handbook no. 15. Blackwell, Oxford.
23. **Wanner, B.** Personal communication.
24. **Wanner, B. L.** 1990. Phosphorus assimilation and its control of gene expression in *Escherichia coli*, p. 152–163. *In* G. Hauska and R. Thauer (ed.), The molecular basis of bacterial metabolism. Springer-Verlag KG, Berlin.
25. **Zhan, H., and J. A. Leigh.** 1990. Two genes that regulate exopolysaccharide production in *Rhizobium meliloti*. J. Bacteriol. **172**:5254–5259.
26. **Zhan, H., S. B. Levery, C. C. Lee, and J. A. Leigh.** 1989. A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. USA **86**:3055–3059.