APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4978–4981 Vol. 63, No. 12
0099-2240/97/$04.00+0

Regulation of the Phosphate Stress Response in *Rhizobium meliloti* by PhoB†

THAMIR S. AL-NIEMI,[1] MICHAEL L. SUMMERS,[1] JAMES G. ELKINS,[1]
MICHAEL L. KAHN,[2] AND TIMOTHY R. McDERMOTT[1]*

Department of Plant, Soil, and Environmental Sciences, Montana State University, Bozeman, Montana 59717-0312[1] and Institute of Biological Chemistry and Department of Microbiology, Washington State University, Pullman, Washington 99164-6340[2]

Received 7 May 1997/Accepted 21 September 1997

Alkaline phosphatase activity and phosphate transport rates in *Rhizobium meliloti* increased significantly when medium phosphate levels decreased to approximately 10 μM. Both responses were abolished in a Tn*5*::*phoB* mutant, but the mutant could be complemented by a plasmid that contained cloned *R. meliloti phoB*. The PhoB$^-$ mutant had a normal symbiosis phenotype under growth conditions that supplied either limiting or nonlimiting levels of phosphate to the host plant *Medicago sativa*, suggesting that induction of genes by PhoB was not required for normal symbiotic function.

Legume plants obtaining nitrogen (N) from symbiosis require higher levels of phosphate (P) for optimal growth than legumes grown with N fertilizers (14). Virtually every aspect of the symbiosis is significantly enhanced by P addition (7, 14, 15, 20, 21). The physiological basis for this P response is not understood, and little information regarding bacteroid P metabolism and acquisition is available. Free-living rhizobia respond to P stress by increasing alkaline phosphatase (AP) activity and P transport rates (1, 4, 24, 25). These changes resemble those seen in *Escherichia coli* (reviewed in reference 27) with respect to responses to P limitation. In *E. coli* (27), control of the PHO regulon is primarily governed by the two-component regulatory pair PhoR-PhoB (17, 27); PhoR is the sensor, and PhoB is the cognate regulatory protein. When environmental P becomes limiting, PhoR transduces this signal by phosphorylating PhoB, and the phosphorylated PhoB then activates transcription of genes in the PHO regulon. The PHO regulon does not respond to P limitation in PhoB$^-$ mutants (27).

AP is present at a high level in *Rhizobium tropici* bacteroids in P-sufficient bean plants, suggesting that inorganic P levels in the bean symbiosome are low enough to trigger the P stress response (1). A *Rhizobium meliloti* mutant lacking a functional inorganic P transporter has a severe nodulation phenotype (4), implying that at least during nodule initiation, rhizobia take up P as the inorganic ion. It is not known if basal-level expression of this P transporter is adequate for P acquisition during nodule formation or if higher-level expression is required. The objectives of the present work were to study the basic responses of *R. meliloti* to P stress, to determine the P level at which *R. meliloti* induces its P stress response, and to isolate an *R. meliloti phoB* mutant and assess its symbiotic phenotype as a means of determining if up-regulation of the PHO regulon is required for normal symbiosis.

Response of wild-type *R. meliloti* to P limitation. The minimal mannitol-ammonium chloride medium ($MMNH_4$) described by Sommerville and Kahn (26) was modified by replacing the phosphate buffer with 5 mM morpholineethanesulfonic acid (MES) and 10 mM morpholinepropanesulfonic acid (MOPS), and this medium was supplemented with P as needed. *R. meliloti* 104A14 (26) was grown to mid- to late log phase in $MMNH_4$ containing 5 mM P and then washed and resuspended in $MMNH_4$ containing no P or $MMNH_4$ containing 5 mM P. The AP activity and P transport rate were measured at intervals (Fig. 1). After 8 h of P starvation, AP activity had increased 20-fold to 4.5 ± 0.3 nmol · min^{-1} · $A_{600}{}^{-1}$, and P transport had increased 6-fold to 14 ± 0.5 nmol of P · min^{-1} · mg of cell dry weight^{-1} (results from three separate experiments each). Thus, AP could be used as a marker enzyme for the induction of P-sensitive genes.

P depletion experiments were conducted to determine the P concentration at which AP was induced. Washed cells were resuspended in $MMNH_4$ containing 90 μM P, and the P concentration in the medium and AP activity were monitored during subsequent incubation (Fig. 2). As the medium P concentration decreased to approximately 10 μM, AP activity rapidly increased. By contrast, in *R. tropici* AP does not induce until medium P levels are approximately 1 μM (1). Extracellular polysaccharide production increases in *R. meliloti* in response to P limitation (6, 28) and may contribute to small changes in optical density, but in our experiments and in *R. tropici* (1), culture optical density correlated well with limited cell division, as determined by viable cell counts (11).

Identification of a *phoB* mutant. Transposon Tn*5* was conjugated from *E. coli* S17-1 (23) into *R. meliloti* via the suicide vector pSUP2021 (9). Putative *phoB* mutants were identified by first screening for loss of AP activity on $MMNH_4$ agar containing 60 μM P and kanamycin (50 μg · ml^{-1}). Plates were flooded with a filter-sterilized solution containing 30 mM Tris (pH 8.5), 50 mM $MgCl_2$, and 0.4% *p*-nitrophenylphosphate, a chromogenic phosphatase substrate. Colonies of putative mutants were white, whereas transconjugants that were wild type for AP activity stained an intense yellow. In subsequent experiments, one AP$^-$ mutant, RmMSU3, was unable to induce either AP (Fig. 3) or higher P transport rates (Fig. 4) under P limitation conditions. Extending P starvation to 14 h also failed to elicit induction of AP. The lack of inducible AP levels or increased P transport rates under P-limiting conditions was preliminary evidence that the mutation in RmMSU3 affected the regulation of the phosphorus stress response.

The single transposon and flanking DNA from RmMSU3

* Corresponding author. Phone: (406) 994-2190. Fax: (406) 994-3933. E-mail: timmcder@terra.oscs.montana.edu.

† Montana State Agricultural Experiment Station Contribution J-5156.

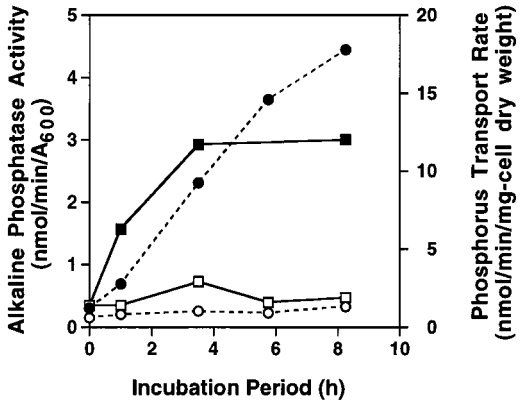

FIG. 1. Response of *R. meliloti* wild-type strain 104A14 to P stress. Symbols: ○ and □, cells grown with P; ● and ■, cells grown without P; ○ and ●, AP activity; □ and ■, P transport. P transport was measured as described by Al-Niemi et al. (1). AP activity was measured with whole cells by using *p*-nitrophenylphosphate as the substrate (1). The culture turbidity coefficient was calculated as follows: $y = 0.4024x + 0.0144$, where y is the cell dry weight and x is the culture optical density at 600 nm. The results shown are the results from one of three experiments in which after 6 h of incubation in P media lacking P the induced levels of AP and P transport always exceeded the constitutive levels observed with cells grown in the presence of P by at least 10-fold and 5-fold, respectively.

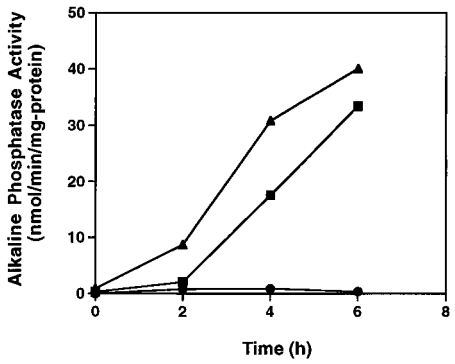

FIG. 3. Increase in AP activity after P starvation depends on PhoB. Mid-log-phase cells of *R. meliloti* 104A14 (wild type) (■), the *phoB* mutant RmMSU3 (●), and RmMSU3 complemented by the wild-type *phoB* allele carried in pTA5 (▲) were washed and resuspended in $MMNH_4$ containing no P and then incubated for up to 6 h. In these experiments, AP activity was measured in periplasmic extracts to demonstrate the reproducibility and reliability of the whole-cell assay technique used to obtain the data in Fig. 1 and 2. Periplasmic protein extraction, protein concentration measurement, and AP assays were performed as described by Al-Niemi et al. (1). These experiments were reproduced three times, and after 6 h of incubation under P stress conditions, the AP specific activities in periplasmic extracts of 104A14 and RmMSU3(pTA5) were found to be at least 15-fold greater than the AP activities in RmMSU3 or cells incubated in rich P-containing media (2).

were cloned as an *Eco*RI fragment into pBlueScript (Stratagene) by using standard methods (22) and were transformed into *E. coli* DH5α (22). The DNA sequence was determined in *Eco*RI-*Bam*HI subclones by the dideoxy chain termination method by using Sequenase II T7 polymerase (United States Biochemical) and the Tn*5* sequence 5′-ATGCTGCAGTCAG TC-3′ as a primer to read into DNA adjacent to the transposon. Sequences obtained from these reactions were used to design commercially prepared primers to sequence the opposite strand. Homology searches of major databases (3) and sequence alignments (10) revealed that the 150 bp of sequence adjacent to the transposon was 93% identical to *R. meliloti* Rm1021 (19) *phoB* (GenBank accession no. M96261) at the nucleotide level, and the inferred peptide sequence was 100% identical (2). Alignments of the deduced peptide sequence with the sequences of other gram-negative bacteria revealed that the deduced peptide sequence exhibited 62% identity and 80% similarity with *E. coli* PhoB and 56% identity and 76% similarity with *Pseudomonas aeruginosa* PhoB. The transposon insert site was bp 279 and 280 in the coding region of the 681-bp Rm1021 *phoB* gene.

Cloning of *phoB* and complementation of the PhoB⁻ mutant. A 720-bp fragment was cloned from *R. meliloti* Rm1021 by using PCR (13) (the sense primer was 5′-GCTGTCGTGC GTAGGAAGGAGT-3′ and was located in the 5′-untranslated region of *phoB*; the antisense primer was 5′-CGGCAC CTTCGGTCTCAGCTCTCC-3′ and extended to within three amino acids of the C terminus of *phoB*), and this fragment was used as a probe in colony hybridizations with a *Hin*dIII partial 104A14 subgenomic library (constructed in pBlueScript [Strat-

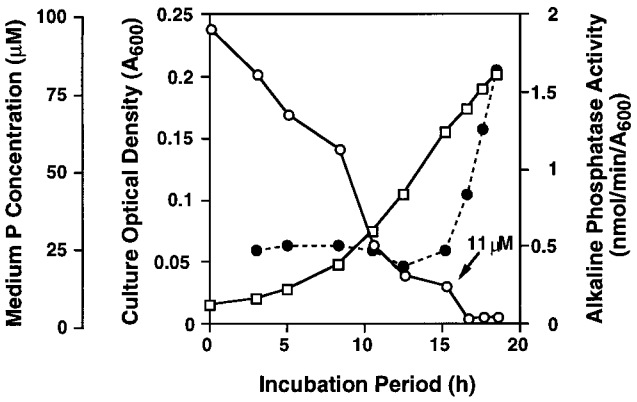

FIG. 2. AP activity is induced as medium P is depleted. Mid-log-phase cells were washed and resuspended in $MMNH_4$ containing 90 μM P at time zero. Culture optical density (□), whole-cell AP activity (●), and medium P concentration (○) were measured at the times indicated. The P concentration in the spent medium was measured by the method of Chen et al. (8). The results shown are from one of three separate experiments that demonstrated induction of AP when the medium P concentration decreased to approximately 10 μM.

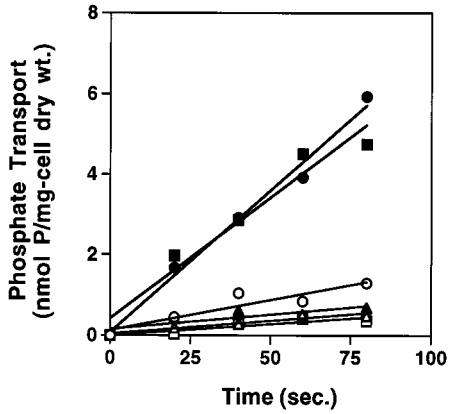

FIG. 4. Increase in P transport rate after P starvation depends on PhoB. Cells were washed in $MMNH_4$ containing no P, resuspended in either $MMNH_4$ containing no P (solid symbols) or $MMNH_4$ containing 5 mM P (open symbols), and incubated for an additional 6 h. Cells were then washed and resuspended in $MMNH_4$ containing no P and supplemented with chloramphenicol (50 μg · ml^{-1}) to stop protein synthesis, and then P transport was measured. *R. meliloti* wild-type strain 104A14 (● and ○) and *phoB* mutant strain RmMSU3 with (■ and □) or without (▲ and △) the cloned *phoB* allele carried on plasmid pTA5. Similar results were obtained in two other experiments.

TABLE 1. Symbiotic phenotypes of *R. meliloti* 104A14 and PhoB$^-$ mutant RmMSU3

P level	Strain	Symbiotic responses[a]			
		Shoot dry wt (mg plant^{-1})	No. of nodules plant^{-1}	Nodule fresh wt (mg plant^{-1})	Acetylene reduction (μmol · h^{-1} · g of nodules^{-1})
Low	104A14	7.2 B	6.7 A	4.3 A	11.1 A
	RmMSU3	7.3 B	6.4 A	4.3 A	11.3 A
	Uninoculated control	4.1 A			
High	104A14	16.7 C	8.3 A	7.0 B	8.5 A
	RmMSU3	15.2 C	7.2 A	7.0 B	9.3 A
	Uninoculated control	3.7 A			

[a] The beginning equilibrium phosphorus levels were 2 to 5 and $\geq$25 μM for the low- and high-phosphorus treatments, respectively. Plants were harvested after 6 weeks of culture in a growth chamber at 25°C with a light intensity of approximately 400 μmol · m^2 · s^{-1} and a photoperiod consisting of 16 h of light and 8 h of darkness. Acetylene reduction was estimated as described by McDermott and Kahn (18). The data shown are the means from at least five replications for each treatment. The means in each column followed by the same letter are not significantly different ($LSD_{\alpha=0.05}$).

agene]). A hybridizing 2.3-kb *Hin*dIII fragment was identified and subcloned as a *Kpn*I-*Xba*I fragment into broad-host-range plasmid pCPP30 (5) to create plasmid pTA5. pTA5 was transformed into *E. coli* S17-1 and then mobilized into RmMSU3 by conjugation (selection with tetracycline [10 μg · ml^{-1}]). The PHO regulatory phenotype of RmMSU3 was corrected by pTA5, as shown by induction of both AP (Fig. 3) and increased rates of P transport (Fig. 4) in response to P limitation. The *R. meliloti* 104A14-specific primers used for sequencing of the Tn*5*-chromosome junctions were also used to confirm the presence and sequence of the *phoB* gene in the complementing *Hin*dIII fragment (2).

Symbiosis phenotype of the PhoB$^-$ mutant. The symbiosis phenotype of the *phoB* mutant was determined by infecting alfalfa seedlings in sterile growth box units with washed suspensions of 104A14 and RmMSU3 by using axenic plant growth conditions as described by McDermott and Kahn (18), except that the sand was replaced by a mixture of sand and P-loaded alumina (12). When this technique is used, plants can be cultured with various amounts of P, and we were able to ask if PhoB-controlled gene expression was required when the host plant alfalfa was stressed for phosphate.

Higher phosphate concentrations increased plant dry matter production and nodule fresh weight in plants infected with either 104A14 or RmMSU3 (Table 1). The number of nodules per plant also increased under high-P conditions, but the differences were not statistically significant. The data suggests that average nodule size increased as a consequence of additional P, but this apparently did not translate into higher nitrogen fixation rates as specific nodule acetylene reduction rates did not differ between P treatments. There were no significant differences in any of the parameters studied between the wild-type and mutant strains at either P level. One hundred isolates obtained from nodules formed by RmMSU3 were all resistant to kanamycin (25 μg · ml^{-1}); 20 of these were tested and found to be unable to induce AP in response to P stress. These results indicated that the Fix$^+$ phenotype was not due to transposon instability.

The starting P concentration in the low-P plant growth experiments was less than 5 μM and therefore below the P concentration at which the PhoB-directed P stress response was induced (Fig. 2). Assuming that the P concentration in the rhizosphere was similarly low and PhoB-regulated gene expression in the rhizosphere occurs normally in response to low P levels, the lack of a nodulation phenotype in RmMSU3 in the low-P plant growth experiments implies that there is no important regulatory interaction between PhoB and nodulation genes. Also, under both high- and low-P plant growth conditions, nodules formed by RmMSU3 were Fix$^+$, suggesting that loss of a functional PhoB also does not disturb the symbiotic function of *R. meliloti*, regardless of the P available for the host plant. Bardin et al. (4) showed that an inorganic P transporter was necessary for effective symbiosis. Our results suggest that PhoB-induced expression of this transporter is not needed to provide sufficient P for normal nodule development. It is possible that basal levels of transporter expression are sufficient or that other regulatory systems might induce the PHO regulon of *R. meliloti* in the absence of PhoB. *Agrobacterium tumefaciens* ChvI has been shown to complement an *E. coli* PhoB$^-$ mutant (16), even though an *A. tumefaciens chvI* mutant did not have a PHO phenotype (16). Therefore, it is possible that the ChvI homolog in *R. meliloti* (19) may substitute for PhoB such that up-regulation of PHO genes during symbiosis, if required, is maintained in RmMSU3. We are currently investigating this possibility.

This work was initiated while T.R.M. was a National Science Foundation Postdoctoral Fellow in Plant Biology (supported by grant BIR-9203796) in the laboratory of M.L.K. This research was funded by grant 94-37305-0574 to T.R.M. from the U.S. Department of Agriculture and by grant IBN-9420798 to T.R.M. from the National Science Foundation. The work contributed by J.G.E. was supported by starter grant IBN-9413485 to T.R.M. from the National Science Foundation.

We thank Lina Botero for technical assistance.

REFERENCES

1. **Al-Niemi, T. S., M. L. Kahn, and T. R. McDermott.** 1997. Characterization of phosphorus metabolism in the bean-*Rhizobium tropici* symbiosis. Plant Physiol. **113:**1233–1242.
2. **Al-Niemi, T. S., M. L. Summers, and T. R. McDermott.** Unpublished data.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. L. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Bardin, S., S. Dan, M. Osteras, and T. M. Finan.** 1996. A phosphate transport system is required for symbiotic nitrogen fixation by *Rhizobium meliloti*. J. Bacteriol. **178:**4540–4547.
5. **Bauer, D.** Unpublished data.
6. **Breedveld, M. W., A. J. Benesi, M. L. Marco, and K. J. Miller.** 1995. Effect of phosphate limitation of synthesis of periplasmic cyclic β-(1,2)-glucans. Appl. Environ. Microbiol. **61:**1045–1053.
7. **Cassman, K. G., A. S. Whitney, and R. L. Fox.** 1981. Phosphorus requirements of soybean and cowpea as affected by mode of N nutrition. Agron. J. **73:**17–22.
8. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorus. Anal. Chem. **28:**1756–1758.
9. **de Bruijn, F. J., and J. R. Lupski.** 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
10. **Devereux, J., V. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
11. **Elliot, B., and T. R. McDermott.** Unpublished data.
12. **Gourley, C. J. P., D. L. Allen, M. P. Russelle, and P. R. Bloom.** 1993. Evaluation and improvements of a sand-alumina culture technique to screen

plants for low phosphorus tolerance. Soil Sci. Soc. Am. J. **57:**103–110.
13. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innes, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, San Diego, Calif.
14. **Israel, D. W.** 1987. Investigation of the role of phosphorus in symbiotic dinitrogen fixation. Plant Physiol. **84:**835–840.
15. **Itoh, S.** 1987. Characteristics of phosphorus uptake of chickpea in comparison with pigeon pea, soybean and maize. Soil Sci. Plant Nutr. **33:**417–422.
16. **Mantis, N. J., and S. C. Winans.** 1993. The chromosomal response regulatory gene *chvI* of *Agrobacterium tumefaciens* complements an *Escherichia coli phoB* mutation and is required for virulence. J. Bacteriol. **175:**6626–6636.
17. **McCleary, W. R.** 1996. The activation of PhoB by acetylphosphate. Mol. Microbiol. **20:**1155–1163.
18. **McDermott, T. R., and M. L. Kahn.** 1992. Cloning and mutagenesis of the *Rhizobium meliloti* isocitrate dehydrogenase gene. J. Bacteriol. **174:**4790–4797.
19. **Osteras, M., J. Stanley, and T. M. Finan.** 1995. Identification of *Rhizobium*-specific intergenic mosaic elements within an essential two-component regulatory system of *Rhizobium* species. J. Bacteriol. **177:**5485–5494.
20. **Pereira, P. A. A., and F. A. Bliss.** 1987. Nitrogen fixation and plant growth of common bean (*Phaseolus vulgaris* L.) at different levels of phosphorus availability. Plant Soil **104:**79–84.
21. **Powell, C. L.** 1977. Mycorrhizas in hill country soils. III. Effect of inoculation on clover growth in unsterile soils. N.Z. J. Agric. Res. **20:**343–348.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
24. **Smart, J. B., A. D. Robson, and M. J. Dilworth.** 1984. A continuous culture study of the phosphorus nutrition of *Rhizobium trifolii* WU95, *Rhizobium* NGR 234 and *Bradyrhizobium* CB756. Arch. Microbiol. **140:**276–280.
25. **Smart, J. B., M. J. Dilworth, and A. D. Robson.** 1984. Effect of phosphorus supply on phosphate uptake and alkaline phosphatase activity in rhizobia. Arch. Microbiol. **140:**281–286.
26. **Somerville, J. E., and M. L. Kahn.** 1983. Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. J. Bacteriol. **156:**168–176.
27. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
28. **Zhan, H., C. C. Lee, and J. A. Leigh.** 1991. Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations. J. Bacteriol **173:**7391–7394.