

Regulation of Phosphate Assimilation in *Rhizobium (Sinorhizobium) meliloti*

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ABSTRACT

We report the isolation of *phoB* and *phoU* mutants of the bacterium *Rhizobium (Sinorhizobium) meliloti*. These mutants form N₂-fixing nodules on the roots of alfalfa plants. *R. meliloti* mutants defective in the *phoCDET* (*ndvF*) encoded phosphate transport system grow slowly in media containing 2 mM Pi, and form nodules which fail to fix nitrogen (Fix⁻). We show that the transfer of *phoB* or *phoU* insertion mutations into *phoC* mutant strains restores the ability of these mutants to: (i) form normal N₂-fixing root-nodules, and (ii) grow like the wild type in media containing 2 mM Pi. We also show that expression of the alternate *orfA pit* encoded Pi transport system is negatively regulated by the *phoB* gene product, whereas *phoB* is required for *phoCDET* expression. We suggest that in *R. meliloti* cells growing under Pi limiting conditions, PhoB protein activates *phoCDET* transcription and represses *orfA pit* transcription. Our results suggest that there are major differences between the *Escherichia coli* and *R. meliloti* phosphate regulatory systems.

THE presence of two phosphate transport systems is a common feature in bacteria, and such systems have been identified in *Escherichia coli* (Willisky and Malamy 1980), *Acinetobacter johnsonii* (Van Veen *et al.* 1993a), and *Bacillus cereus* (Rosenberg *et al.* 1969). Differences in specificity and affinity for phosphate between the two Pi transporters, as observed in *E. coli* and *A. johnsonii* (Van Veen *et al.* 1993b, 1994) suggest that the organisms have evolved two Pi transport systems in order to adjust to the different phosphate concentrations found in their environment.

The high-affinity phosphate-specific transport system of *E. coli*, PstSCAB, is a periplasmic-binding protein, ABC-type transporter, and *pstSCAB* transcription is induced when cells are starved for phosphate (Wanner 1996). Induction of the *pstSCAB* genes is regulated by the PhoB and PhoR proteins, which constitute a two-component regulatory system (Ronson *et al.* 1987; Parkinson 1993; Tommassen *et al.* 1982). The environmental sensor histidine kinase protein, PhoR, autophosphorylates under low phosphate conditions and then phosphorylates PhoB; in high phosphate conditions, PhoR probably dephosphorylates phospho-PhoB (Makino *et al.* 1989, 1994). Phosphorylated PhoB binds to DNA sequences, called Pho boxes, which are found in the -35 promoter region of PhoB-regulated genes (Makind *et al.* 1994, 1996). Under Pi limiting conditions, PhoB activates transcription of a number of genes, which together constitute the Pho regulon. These include the *pstSCAB* genes and the *phoA* gene encoding alkaline phosphatase.

In *E. coli*, the PstSCAB and PhoU proteins appear to be involved in the sensing of environmental Pi, as many *pstSCAB phoU* mutations result in constitutive expression of the Pho regulon (Amemura *et al.* 1982; Cox *et al.* 1981). On the other hand, Pi transport *per se* does not appear to be a sensory signal, as one PstA and two PstC missense mutations which abolish Pi transport show wild-type regulation of the Pho regulon (Cox *et al.* 1988, 1989). Steed and Wanner (1993) showed that the *phoU* gene of *E. coli* is not required for Pi transport. However, *phoU* mutants grew poorly, suggesting that PhoU has another function in addition to its role in regulation of the Pho regulon.

Wild-type *Rhizobium meliloti* form N₂-fixing nodules on alfalfa, whereas mutants defective in the *phoCDET* (originally designated *ndvF*) encoded phosphate transport system form nodules which contain few bacteria and fail to fix N₂ (Fix⁻) (Bardin *et al.* 1996; Charles *et al.* 1991). The Fix⁻ phenotype of *phoCDET* mutants can be suppressed to Fix⁺ by spontaneous mutations which have been separated into two genetic classes, designated I and II (Oresnik *et al.* 1994). The Class I suppressor allele, *sfx1*, was shown to map to the promoter region and increase transcription of an operon, *orfA-pit*, which encodes a second phosphate transport system, and we hypothesized that suppression of the *phoCDET*Fix⁻ phenotype resulted from increased phosphate uptake via the Pit system (S. D. Bardin, R. Voegelé, N. Falcioni and T. M. Finan, unpublished results; Voegelé *et al.* 1997).

In this article, we report the isolation of *phoU* and *phoB* mutants of *R. meliloti*. The *phoU/B* mutations are shown by transduction to map to the Class II suppressor locus (*sfx2*) and also to suppress the Fix⁻ phenotype of *phoCDET* mutants to Fix⁺. We show that *phoB* is required for

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phoCDET expression, and that *phoB* regulates expression of the *orfA-pit* operon in a phosphate-dependent manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions:

The strains and plasmids employed in this work are listed in Table 1. The growth media used were Luria-Bertani (LB) or LB containing 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc) with antibiotic concentrations as previously described (Charles and Finan 1991). The phosphate-free media was morpholino-propane sulfonic acid (MOPS)-buffered minimal media described in Bardin *et al.* (1996), except this medium was supplemented with 14 μ l/liter of a yeast extract fraction which stimulates growth of *R. meliloti* in defined medium (Bob Watson, personal communication). For the growth experiments, 24-hr LBmc cultures were washed and resuspended in phosphate-free MOPS media (MOPS P0). Twenty microliters of these cells were used to inoculate 5 ml of MOPS P0 (OD₆₀₀ ~0.05). The cells were grown for 24 hr under agitation. The resulting culture densities were adjusted to an OD₆₀₀ of 0.2, and 5 μ l were then used to inoculate 5 ml of MOPS P0 and MOPS supplemented with 2 mM ortho-phosphate (MOPS P2) or 2 mM aminoethylphosphonate (AEP) (Sigma Chemical Co., St. Louis) media. The phosphate starvation step reduced the cellular phosphate reserves (presumably polyphosphate), which otherwise allowed significant growth in MOPS P0 media.

For experiments employing *lacZ* gene fusions, LBmc-grown cells, supplemented with tetracycline (Tc) 2 μ g/ml for strains carrying pMP220-derived plasmids (Spaink *et al.* 1987), were washed once and resuspended in MOPS P0. 20 μ l and 5 μ l of these cells were then used to inoculate 5 ml of MOPS P0 and MOPS P2 media, respectively. These cultures were grown for 38 hr (OD₆₀₀ reaching 0.3–0.4 in MOPS P0, and 0.8–1 in MOPS P2 media) before performing the β -galactosidase and alkaline phosphatase (AP) assays.

The low osmolarity media (GYM) was prepared as stated in Oresnik *et al.* (1994). The AP phenotype of colonies was determined on solid LB agar medium containing 60 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate (LBXPhos). AP⁺ colonies are blue, AP⁻ colonies are white.

The chromosomal transcriptional *lacZ* fusions to *pit* were constructed as outlined in Figure 8. The structure of the recombinants was confirmed by Southern blot of the genomic DNA digested with *Hind*III, *Sac*I, and *Hind*III/*Sac*I, and probed with the 4.8-kb *Hind*III/*Sac*I fragment of pTH90. All β -galactosidase values reported in Figure 6 were corrected for the β -galactosidase activity obtained from recombinants with the *lacZ* gene in the opposite orientation (ort II) to the direction of *orf-pit* transcription (*e.g.*, RmH695). This background activity remained constant at 10 Miller Units, regardless of the background or whether the fusion was to the *sfx1* or wild-type locus.

DNA manipulation and genetic techniques: Cloning procedures, including DNA isolation, restriction digests, ligation, and transformation were performed according to Sambrook *et al.* (1989).

Conjugal mating using MT616 as a helper strain, Φ M12 generalized transduction, TnV and Tn5-233 replacement, and determination of the linkage between two markers by transduction were performed as described previously (Charles and Finan 1991; Finan *et al.* 1988, 1986, 1984).

TnV possesses the NptII gene (Km^r) and the pSC101 origin of vegetative replication (able to replicate in *E. coli* but not in *R. meliloti*), flanked by the inverted repeats IS50_L and IS50_R (Furuichi *et al.* 1985). Following the replacement of the *pho10*, *pho8*, and *pho3*Tn5-132 insertions with TnV, total genomic DNA from these strains was digested with *Sal*I, religated,

and transformed into DH5 α -competent cells. As TnV does not contain a *Sal*I restriction site, the religated plasmid carried flanking genomic DNA. Subclones of the resulting plasmids were sequenced using the IS50 primer (5'-TCACATGGAAGT CAGATCCT-3').

Plant growth, alkaline phosphatase, and β -galactosidase assays:

Plant growth experiments in a nitrogen-free environment and acetylene reduction assays were performed as stated in Charles *et al.* (1991), except that a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph (air 34 psi, H₂ 12 psi, N₂ 65 psi) was used, and the ethylene peaks were integrated using the HP3365 (Hewlett-Packard) Series II Chemstation computer program. Plant dry weight was determined by weighing the plant shoots of one pot, dried for one week in an oven, and dividing by the number of plants.

The alkaline phosphatase activity was measured as described in Charles *et al.* (1991), except that the cells were spun down before the OD₄₂₀ was measured. The alkaline phosphatase activity was calculated using the formula (1000 \times OD₄₂₀) / (OD₆₀₀ \times Δ T), with Δ T being the reaction time (min).

The β -galactosidase assay was performed by mixing 0.5 ml of cell, for which OD₆₀₀ was determined, with 0.5 ml Buffer Z (pH 7; 60 mM Na₂HPO₄; 40 mM NaH₂PO₄; 10 mM KCl; 1 mM MgSO₄ and 2.7 ml/liter of 2-mercaptoethanol added just before use), 20 μ l chloroform and 10 μ l 0.1% SDS. The tubes were equilibrated at 30° for 5 min, and the reaction was started by adding 0.2 ml of 4 mg/ml *o*-nitrophenyl- β -D-galactoside (ONPG). When the solution turned yellow, the reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃. The optical density at 420 nm (OD₄₂₀) was determined after centrifuging the cells for 5 min at 12,000 rpm. The β -galactosidase activity in Miller Units was calculated using the formula (1000 \times OD₄₂₀) / (OD₆₀₀ \times Δ T \times V), with Δ T being the reaction time (min) and V representing the initial volume of culture used (ml).

RESULTS

Strains carrying *sfx2* are deficient in alkaline phosphatase production: Measurements of alkaline phosphatase activity in *R. meliloti* cells can be used to monitor the physiological status of cultures with respect to phosphate availability (Bardin *et al.* 1996). Thus, the level of AP activity detected in Rm1021 wild-type cells cultured in MOPS-buffered minimal media with no added phosphate (Pi 0 mM) is 10–20-fold higher than the AP activity found in cells grown in the same media containing 2 mM inorganic phosphate (Pi 2 mM) (Figure 1). Similar measurements for cells of the *R. meliloti phoC* mutant, RmG490, revealed a high AP activity even in media containing 2 mM Pi (Figure 1). Thus, even in the presence of 2 mM Pi, RmG490 cells appeared to be starved for phosphate. This phenotype is consistent with the observation that RmG490 grows poorly in media containing 2 mM Pi (S. D. Bardin, R. Voegelé, N. Falconi and T. M. Finan, unpublished results). As we had previously found that *R. meliloti phoCDET* mutants grew like the wild type in MOPS media containing 2 mM AEP as sole source of phosphorus (Bardin *et al.* 1996), we measured AP activity in cultures of RmG490 and the wild-type Rm1021 following growth in this medium (Figure 1). Both RmG490 and wild-type cells contained background AP activities after growth with 2 mM AEP.

TABLE 1
Bacterial strains and plasmids

Strains/plasmids	Relevant characteristics	Origin
<i>R. meliloti</i>		
Rm1021	SU47, <i>str-21</i>	Charles <i>et al.</i> 1991
RmF114	$\Delta\Omega 5033$ -5064 (<i>phoCDET</i>)::Tn5-233, Fix ⁻	Charles and Finan 1991
RmF346	$\Delta\Omega 5033$ -5064 (<i>phoCDET</i>)::Tn5-233 <i>sfx2</i> , Fix ⁺	Oresnik <i>et al.</i> 1994
RmG212	<i>lac</i> ⁻	J. Glazebrook, MIT
RmG425	$\Delta\Omega 5033$ -5064 (<i>phoCDET</i>)::Tn5-233 <i>sfx3</i> , Fix ⁺	Oresnik <i>et al.</i> 1994
RmG439	$\Delta G439$ [Δ <i>phoCDET HindIII</i> ::Nm ^r (12 kb)] = $\Delta G439(\Delta$ <i>phoCDET</i>) Fix ⁻	Charles <i>et al.</i> 1991
RmG490	<i>ndvF</i> 1.7 Ω Sp ^r = <i>phoC</i> Ω 490, Fix ⁻	Charles <i>et al.</i> 1991
RmG479	$\Omega 5025$::Tn5 <i>sfx2</i>	Oresnik <i>et al.</i> 1994
RmG497	$\Omega 5033$::Tn5-233 <i>sfx2</i>	Oresnik <i>et al.</i> 1994
RmG514	$\Delta G439(\Delta$ <i>phoCDET</i>), <i>sfx2</i> $\Omega 5025$::Tn5, Fix ⁺	Oresnik <i>et al.</i> 1994
RmG551	Rm1021, <i>sfx1</i>	Finan Laboratory collection
RmG552	$\Delta\Omega 5033$ -5064::Tn5-233 $\Omega 5259$::Tn5	Finan Laboratory collection
RmG591	$\Omega 5262$::Tn5-233 <i>sfx2</i>	Finan Laboratory collection
RmG640	$\Omega 5263$::Tn5-233 <i>sfx2</i>	Finan Laboratory collection
RmH363	<i>phoC</i> $\Omega 490$ <i>sfx2</i> , Fix ⁺	This work
RmH615	<i>lac</i> ⁻ , Ω <i>phoB3</i> ::TnV	This work
RmH616	<i>lac</i> ⁻ , Ω <i>phoB8</i> ::TnV	This work
RmH617	<i>lac</i> ⁻ , Ω <i>phoU10</i> ::TnV	This work
RmH623	<i>phoC</i> $\Omega 490$, Ω <i>phoU10</i> ::TnV, Fix ⁺	This work
RmH624	<i>phoC</i> $\Omega 490$, Ω <i>phoB8</i> ::TnV, Fix ⁺	This work
RmH625	<i>phoC</i> $\Omega 490$, Ω <i>phoB3</i> ::TnV, Fix ⁺	This work
RmH662	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i>	This work
RmH695	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> , ort II	This work
RmH754	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i> , Ω <i>phoU10</i> ::TnV	This work
RmH755	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i> , Ω <i>phoB8</i> ::TnV	This work
RmH756	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i> , Ω <i>phoB3</i> ::TnV	This work
RmH765	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i> , $\Omega 5258$::Tn5, <i>sfx2</i>	This work
RmH766	<i>lac</i> ⁻ , (wt) <i>pit</i> Ω :: <i>lacZmobSp</i> , $\Omega 5258$::Tn5	This work
RmH771	<i>lac</i> ⁻ , <i>sfx1</i> promoter <i>pit</i> Ω :: <i>lacZmobSp</i>	This work
RmH836	Ω <i>phoU10</i> ::TnV	This work
RmH837	Ω <i>phoB8</i> ::TnV	This work
RmH838	Ω <i>phoB3</i> ::TnV	This work
RmH859	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> $\Omega 5258$::Tn5	This work
RmH860	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> $\Omega 5258$::Tn5, <i>sfx2</i>	This work
RmH861	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> Ω <i>phoU10</i> ::TnV	This work
RmH862	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> Ω <i>phoB8</i> ::TnV	This work
RmH863	<i>lac</i> ⁻ , <i>sfx1</i> <i>pit</i> Ω :: <i>lacZmobSp</i> Ω <i>phoB3</i> ::TnV	This work
Plasmids		
pTF1	pBR322::TnV; Ap ^r , Nm-Km ^r	Furuichi <i>et al.</i> 1985
pLMS	pUC 18 containing the <i>lacZ mob Sp</i> ^r cassette	M. Hynes, Calgary
pTH90	12-kb <i>HindIII</i> (<i>sfx1</i> locus) in pRK7813	S.D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results
pTH365	2.1-kb <i>EcoRI</i> fragment from <i>sfx1</i> locus in pMP220; <i>sfx1</i> promoter <i>orfA-pit-lacZ</i> fusion	S.D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results
pTH376	2.1-kb <i>EcoRI</i> wild-type fragment in pMP220; wild-type promoter <i>orfA-pit-lacZ</i> fusion	S.D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results

Unless otherwise indicated, all *pit* Ω ::*lacZmobSp* insertions fuse *lacZ* to wild type (wt) or *sfx1 orfA-pit* locus such that *pit* and *lacZ* are cotranscribed. In RmH695, *lacZ* was inserted in the opposite orientation to the *pit* gene. Abbreviations are as follows: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; *lac*, lactose utilization genes; *oriV*, origin of vegetative replication.

Thus, unlike what is observed in *E. coli*, where mutants defective in the high-affinity transport system (*pstSCAB*) show constitutive AP expression (Cox *et al.* 1989), in *R. meliloti*, AP expression in the *phoC* mutant was repressed

when a readily assimilated phosphorus source like AEP was provided in the growth media.

To examine the effect of the previously isolated *phoCDET* (*ndvF*) symbiotic suppressor mutations (Ores-

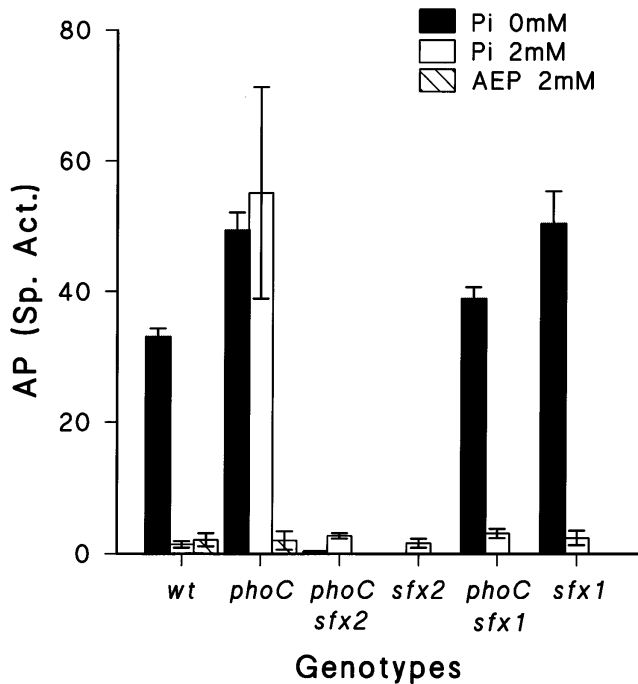


Figure 1.—Alkaline phosphatase (AP) activity of *R. meliloti* strains Rm1021 (wild type), RmG490 (*phoC*), RmH363 (*phoC sfx2*), RmG497 (*sfx2*), RmG762 (*phoC sfx1*), and RmG591 (*sfx1*) after 60 hr growth in MOPS-buffered minimal media containing no added phosphate (MOPS P0, solid box) or 2 mM inorganic phosphate (MOPS P2, open box). The AP activity of Rm1021 and RmG490 was also determined after 80 hr growth in MOPS medium containing 2 mM aminoethylphosphonate (MOPS AEP, hatched box). Each activity represents the average of triplicate values \pm SE.

nik *et al.* 1994) on alkaline phosphatase expression, we measured AP activity in the Class I and Class II suppressor strains RmG762 (*phoC sfx1*) and RmH363 (*phoC sfx2*). AP activity in both these strains was repressed in the presence of 2 mM Pi (Figure 1). Unexpectedly, however, no alkaline phosphatase activity was detected when RmH363 (*phoC sfx2*) was grown in MOPS medium with no Pi added. This result was in contrast with what we observed with the wild type (wt), RmG591 (*sfx1*), and RmG762 (*phoC sfx1*) strains. Subsequent analysis revealed that RmG497 cells that carry the *sfx2* mutation in an otherwise wild-type background, as well as strain RmG425 carrying the other Class II allele, *sfx3*, in a Δ *phoCDET* background, also lacked alkaline phosphatase activity (AP⁻ phenotype) (Figure 1 and data not shown). The AP⁻ phenotype of strains carrying a Class II mutation, together with the suppression of the mucoid colony and symbiotic phenotypes associated with the *phoCDET* (*ndvF*) mutations (Oresnik *et al.* 1994), suggested that *sfx2* and *sfx3* mutations affected a regulatory gene involved in phosphorus assimilation similar to the *phoBR* genes of *E. coli* (Wanner 1993).

Isolation of *phoUB* mutants: To isolate mutants defective in the phosphate-signaling regulatory pathway, we

		Relevant Characteristics
Donor: RmG552, Ω 5259::Tn5		AP ⁺ , Nm ^r , Gm ^r -Sp ^r
Recipient: RmG640, <i>sfx2</i> , Ω 5263::Tn5-233		AP ⁻ , Nm ^s , Gm ^r -Sp ^r

Classes	# in class	Frequency (%)
AP ⁺ , Gm ^r -Sp ^r	17	34
AP ⁺ , Gm ^s -Sp ^s	1	2
AP ⁻ , Gm ^r -Sp ^r	32	64
AP ⁻ , Gm ^s -Sp ^s	0	0

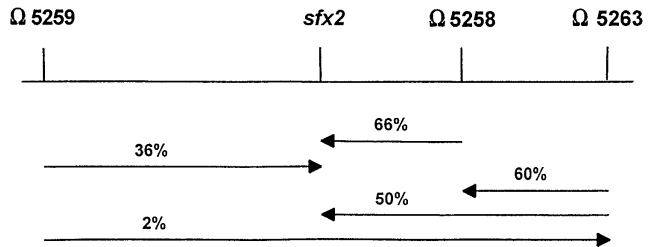


Figure 2.—Genetic linkage map showing the location of two Tn5 insertions (Ω 5258 and Ω 5259) and the Ω 5263::Tn5-233 insertion relative to *sfx2*. The cotransduction frequency is represented in percent, the arrow tail and head refer to the selected and unselected markers, respectively. The table presents the result of a three-factor cross used to determine the order of Ω 5259 relative to *sfx2* and Ω 5263. Neomycin resistance (Nm^r) was transduced from RmG552 into RmG640. 50 transductant colonies selected on LB Nm were screened by patching onto LB Gm-Sp and LBXPhos media. Linkage values to Ω 5258 are based on data not shown. Abbreviations used: AP⁺, produce alkaline phosphatase; AP⁻, deficient in alkaline phosphate production; wt, wild type; (°) and (°), meaning sensitive and resistant, respectively.

screened for alkaline phosphatase negative mutants (AP⁻). Tn5-132 (oxytetracycline resistance) and Tn5 insertion mutants of Rm1021 were plated on LB agar containing the AP chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (LBXPhos). One Tn5 insertion mutant (RmH405, *pho27*) and 26 Tn5-132 mutants which formed white colonies (AP⁻) were identified and purified. To check for linkage of the AP⁻ mutations to the *sfx2* locus, we utilized the insertions Ω 5258::Tn5, Ω 5259::Tn5, and Ω 5263::Tn5-233, which were previously identified to be linked to the *sfx2* locus (Oresnik *et al.* 1994; I. J. Oresnik and T. M. Finan, unpublished results). The order and linkage of these insertions with respect to *sfx2* were deduced from the three-factor cross described in Figure 2.

Nm^r was transduced from strain RmG551 (Ω 5258::Tn5) into the 26 Tn5-132 AP⁻ mutants, and transductants were screened for the presence of blue colonies (AP⁺) on LBXPhos plates. Three of the Tn5-132 insertion mutations designated *pho10*, *pho8*, and *pho3* showed 60, 64, and 66% linkage to Ω 5258::Tn5, respectively. In further transductions, Nm^r from insertion Ω 5259::Tn5 (strain RmG552) showed 48, 44, and 42% linkage to the *pho10*, *pho8*, and *pho3* mutations, respectively. These linkage values were similar to those between the two insertions

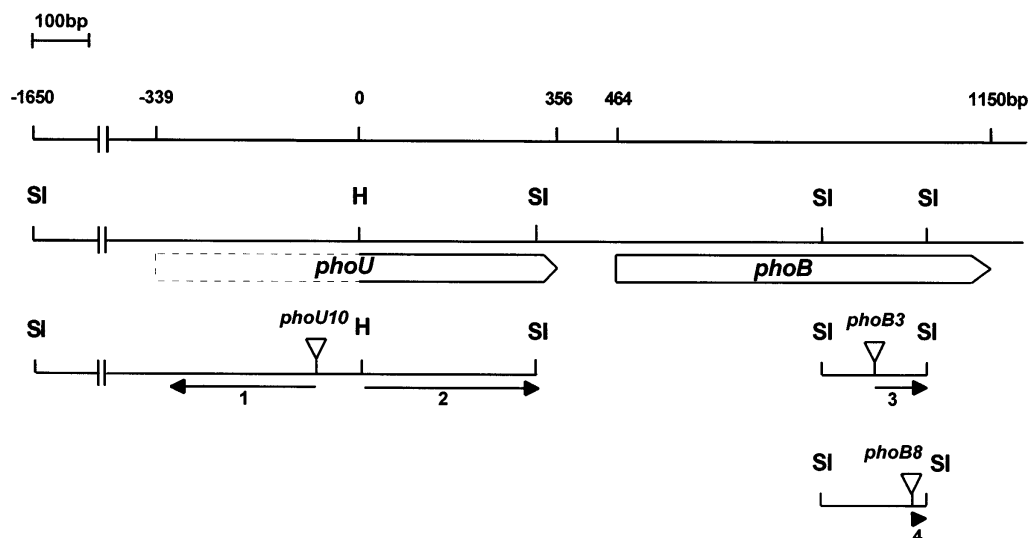


Figure 3.—Map of the *phoU-phoB* locus of *R. meliloti* showing the location of the *phoU10*, *phoB3*, and *phoB8* insertion mutations. The map was drawn from the complete DNA sequence of *phoB* and partial sequence of *phoU* from GenBank (accession number M96261; P. McLean, C. Liu, C. Sookdeo and F. Cannon, unpublished results). The hatched part of the *phoU* gene represents the remaining portion of the gene, as predicted from alignment to the *phoU* gene of *E. coli*. Also indicated are the TnV subclones of *phoU10* (pTH292), *phoB3* (pTH287), and *phoB8* (pTH311), as well as subsequent subclones used for sequencing. The four sequenced fragments (nos. 1–4) are indicated.

Ω 5258 and Ω 5259, and *sfx2*, suggesting that the *pho10*, *pho8*, and *pho3* insertion mutations and *sfx2* map to the same locus (see Figure 2). The remaining 23 AP⁻ (white) Tn5-132 insertion mutants showed no linkage to Ω 5258::Tn5; however, these were 100% linked in transduction to the Tn5 insertion in *pho27* (strain RmH405). These mutants were not examined further.

To identify the gene(s) in which the *pho10*, *pho8*, and *pho3* Tn5-132 insertions were located, we first replaced the Tn5-132 insertions with TnV (Furuichi *et al.* 1985), and then cloned the TnV together with the flanking DNAs as *SalI* fragments into *E. coli*. Following subcloning, the DNA sequence extending into the flanking genomic DNA was determined using a primer which anneals 50 bp inside the end of the IS50 (see materials and methods). Blast searches of the DNA data bases revealed that these sequences were identical with sections of the DNA sequence of the *phoB* and part of the *phoU* genes from *R. meliloti* Rm1021 which have been determined previously (GenBank accession no. M96261, P. McLean, C. Liu, C. Sookdeo, and F. Cannon, unpublished results). A schematic map of the *phoUB* region, together with the locations of the three *pho*:Tn5-132/TnV insertions is shown in Figure 3. The *phoU* and *phoB* genes lie adjacent to one another in the order *phoU-phoB*. The deduced *R. meliloti* PhoB protein is 47.6% identical to the PhoB protein of *E. coli*, while the deduced C-terminal 117 amino acids from PhoU are 36% identical with the corresponding region of the *E. coli* PhoU protein. The *pho10* insertion was located 84 nucleotides into the unsequenced region of *phoU* as determined from sequence alignment to the PhoU protein sequence of

E. coli. Insertions *pho3* and *pho8* were located at position 157 aa and 188 aa of the *R. meliloti* PhoB sequence, respectively. In view of these results, the *pho10*, *pho8*, and *pho3* alleles were designated *phoU10*, *phoB8*, and *phoB3*, respectively.

The location of the *phoUB* locus on the *R. meliloti* genome was determined by conjugation using seven Tn5-*mob* insertion strains as described in Oresnik *et al.* (1994). The Tn5-*mob* insertions were transduced into strain RmG640 (Ω 5263, Tn5-233, 50% linked to *sfx2*; Figure 2). Following purification, the resulting transductants were crossed in conjugation with the rifampicin resistant (Rif^r) Rm5000 strain. The frequency of Rif^r, Gm^r Sp^r colonies placed the *phoUB* locus between *trp-33* and *pyr49* on the *R. meliloti* chromosome (Klein *et al.* 1992). More refined mapping was not carried out.

The *phoUB* genes are required for *phoCDET* expression: We have previously shown that transcription of the *phoCDET* transport genes is induced when *R. meliloti* cells are starved for phosphate, and that the *phoC* promoter contained two elements which are similar in sequence to the consensus-binding site for the *E. coli* PhoB protein (Bardin *et al.* 1996). To determine whether the *phoUB* genes are required for *phoCDET* transcription, plasmids carrying the *phoD7::lacZ* and *phoE19::lacZ* gene fusions (Bardin *et al.* 1996) were transferred into the strain RmG212 (*lac*⁻) and its *phoU* and *phoB* derivatives. β -galactosidase and AP activities were determined in cells cultured both in media without added Pi and with 2 mM Pi added. The AP activity of wild-type and *pho* mutant cells (not shown) was similar to the data shown in Figure 6a. The β -galactosidase activity results clearly showed

that neither *phoD* nor *phoE* were expressed in the *phoB* background (Figure 4, dataset 3). A low level of *phoD* and *phoE* expression was detected in the *phoU* mutant background (Figure 4, dataset 2).

***phoU* and *phoB* mutations suppress the symbiotic and associated phenotypes of *phoCDET* mutants:** The *phoU10*, *phoB8*, and *phoB3* mutations had similar phenotypic effects to the *sfx2* suppressor mutation. As the *sfx2* allele was originally identified as a suppressor of the symbiotic Fix⁻ phenotype of *phoCDET* (*ndvF*) mutants, we tested the three *phoB* and *phoU* insertion mutations for their ability to suppress the Fix⁻ phenotype of a *phoC* mutant. We also examined the ability of *phoB* and *phoU* mutations to suppress the slow growth of *phoCDET* mutants in media containing 2 mM Pi, and the mucoid colony phenotype which *phoCDET* mutants exhibit when plated on low osmolarity media (see below). The *phoUB-phoC* strains were constructed by transducing the *phoU10*, *phoB8*, and *phoB3::TnV* (Nm^r) insertion mutant alleles from strains RmH399, RmH428, and RmH430, respectively, into RmG490 (*phoC*Δ490) to create strains RmH623, RmH624, and RmH625, respectively.

The symbiotic phenotype of the various mutants was determined from the analysis of alfalfa plants 28 days after inoculation with the various *R. meliloti* strains. Plants inoculated with the *phoC* mutant were small and chlorotic, and showed little evidence of N₂-fixation as

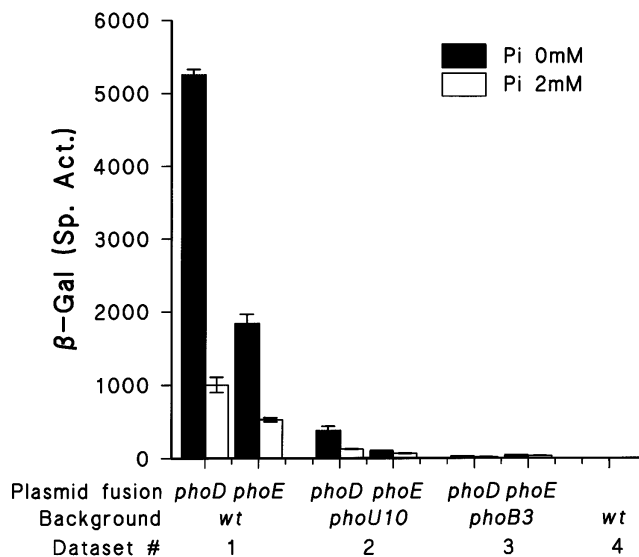


Figure 4.—β-galactosidase activities from plasmid pTH21 *phoD::lacZ* and pTH21 *phoE::lacZ* gene fusions in the following strains: (1) wild type (RmG212), (2) RmH617 (*phoU10*), and (3) Rm615 (*phoB3*). The control dataset 4 shows negligible activity from RmG212 cells lacking the fusion plasmids. The assays were performed after 32 hr growth in MOPS minimal media with no phosphate added (MOPS P0, solid box) or in media supplemented with 2 mM Pi (MOPS P2, open box). Each value corresponds to the mean of triplicate values ±SE. The pTH21 *lacZ* plasmid fusions 7A(*phoD*) and 19(*phoE*) are described in Bardin *et al.* (1996). β-galactosidase activities are expressed as Miller Units.

measured by acetylene reduction and plant dry weight determinations (Table 2). Plants inoculated with the *phoC*, *phoUB* double mutants had shoot dry weight and acetylene reduction values comparable to the wild-type strain Rm1021 (Table 2). Thus, the *phoU10*, *phoB8* and *phoB3* mutations suppressed the Fix⁻ phenotype of the *phoC* to Fix⁺. We also note that the individual *phoB*, and *phoU* insertion mutants showed no reduction in symbiotic effectiveness compared to the wild-type strain.

The ability of the various *R. meliloti* strains to grow in MOPS-buffered minimal media containing 2 mM Pi was determined (Figure 5). While the *phoC* mutant grew poorly, the single *phoB*, *phoU* or *sfx2* mutants, and the double mutants carrying *phoC* together with *phoU10*, *phoB8*, *phoB3* or the *sfx2* allele grew as well as the wild-type strain Rm1021 (Figure 5, data not shown). Given that *phoUB* mutants do not express the *phoCDET* genes (Figure 4), it appears that the transport system(s) which allows Pi uptake into *phoUB* mutants may also be responsible for allowing the *phoUB*, *phoC* double mutants to grow normally in media containing 2 mM phosphate.

Oresnik *et al.* (1994) observed that *phoCDET* (*ndvF*) mutants form mucoid colonies, as opposed to dry wild-type colonies when the strains are plated on low osmolarity GYM medium. Moreover, *phoCDET* *sfx1* and *phoCDET* *sfx2* double mutants formed dry colonies like the wild type on this medium. The mucoid phenotype was dependent on genes required for synthesis of the exopolysaccharide II (EpsII) of *R. meliloti*; these genes were known to be expressed under phosphate starvation conditions (Zhan *et al.* 1991). We investigated whether the *phoUB* mutations were able to suppress the mucoid phenotype of *phoC* mutants by plating RmH623 (*phoC phoU10*), RmH624 (*phoC phoB8*), and RmH625 (*phoC phoB3*) on GYM agar. In all cases, a dry type of colony morphology comparable to that of the wild type was obtained (Table 2). Moreover, when the *phoC* mutant RmG490 was plated on GYM agar supplemented with 2 mM aminophosphonate (a P source the *phoC* mutant strain can utilize), colonies with a dry wild-type morphology were obtained. These data suggest that the mucoid phenotype of *phoCDET* mutants is a direct consequence of the phosphate starvation state of these cells when plated on GYM medium.

In summary, the results presented in this section show that *phoB* and *phoU* mutations suppress the symbiotic and phosphate-dependent phenotypes associated with mutations in the *phoCDET* locus.

Mechanism of *phoUB* mediated suppression of *phoCDET* mutants: In view of the above results, we suspected that *phoB* and/or *phoU* mutations led to increased expression of the recently characterized *orfA-pit* locus, which appears to encode an alternate phosphate transport system (S. D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results). To investigate this possibility, we transduced *phoB*, *phoU*, and *sfx2* mutations into strain RmH662, which carries a chromo-

TABLE 2
Symbiotic and osmotic phenotypes of *R. meliloti* strains

Strain	Genotype	Percent wt ARA	Dry weight (mg/plant) \pm SE	Percent wt dry weight	Colony on GYM
Rm1021	wt	100	39 \pm 5	100	D
RmG490	<i>phoC</i> Ω 490	1	8 \pm 0	19	M
RmG497	<i>sfx2</i>	105	35 \pm 6	90	D
RmH363	<i>phoC</i> Ω 490 <i>sfx2</i>	126	33 \pm 4	84	D
RmH836	<i>phoU10::TnV</i>	141	39 \pm 3	98	D
RmH623	<i>phoC</i> Ω 490 <i>phoU10::TnV</i>	97	39 \pm 3	100	D
RmH937	<i>phoB8::TnV</i>	120	45 \pm 7	115	D
RmH624	<i>phoC</i> Ω 490 <i>phoB8::TnV</i>	109	35 \pm 4	90	D
RmH838	<i>phoB3::TnV</i>	57	39 \pm 1	100	D
RmH625	<i>phoC</i> Ω 490 <i>phoB3::TnV</i>	82	38 \pm 4	98	D
None	—	0	7 \pm 1	17	—

Symbiotic and colony phenotypes of *R. meliloti* wild type, *phoC*, *sfx2*, *phoB*, *phoU*, and *phoC phoB*, *phoC phoU*, and *phoC sfx2* double mutants. Plants were harvested 28 days after inoculation. Acetylene reduction activity (ARA) was determined from the root systems of three plants. Values are the mean of three independent measurements (total of nine roots) \pm SE. The wild-type ARA was 1360 \pm 146 nmol/plant/hr. The shoot weight represents the average of 3 \times 10 plants \pm SE. The percent values are relative to the wild strain (Rm1021). Colony morphology on low osmolarity media was determined by planting the different strains on GYM media. M and D correspond to a mucoid and a dry colony morphology, respectively.

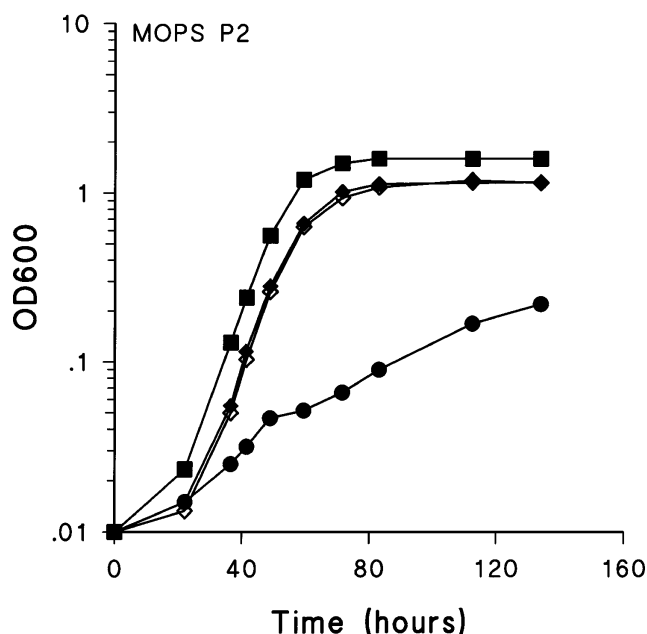


Figure 5.—Growth of *R. meliloti* strains in MOPS medium containing 2 mm Pi. The strains presented are Rm1021 (wt, ■), RmG490 (*phoC*, ●), RmH625 (*phoB3 phoC*, ◇) and RmH838 (*phoB3*, ◆). The growth characteristics of RmH623 (*phoU10 phoC*), RmH624 (*phoB8 phoC*), and RmH363 (*sfx2 phoC*) were similar to the growth of RmH625 and are not presented here to simplify the figure. Each data point represents the mean of triplicate values.

somally located transcriptional *lacZ* fusion to the wild-type *orfA-pit* locus [to give strains RmH754 (*phoU10*), RmH755 (*phoB8*), RmH756 (*phoB3*), and RmH765 (*sfx2*)], and into strain RmH771, which carries the *lacZ* fusion to *sfx1 orfA-pit* locus [to give strains RmH861 (*phoU10*), RmH862 (*phoB8*), RmH863 (*phoB3*), and RmH860 (*sfx2*)]. We note that the *lacZ* fusion construct employed in these experiments was prepared so that *lacZ* was inserted immediately downstream of the *pit* translational stop codon (see materials and methods and Figure 8 for details of this construction). Thus, in the wild-type and *sfx1*-directed gene fusions, both the *orfA*- and *pit*-encoded proteins should be fully functional. (This was confirmed as the *sfx1* constructs in a *phoC* mutant background grew like wild type in MOPS medium containing 2 mm Pi, data not shown.) The level of *orfA-pit* expression (β -galactosidase activity) was measured after culturing the cells for 32 hr in MOPS-buffered medium with no added Pi (P0) and with 2 mm Pi added (Figure 6b). To monitor the physiological status of the cells with respect to phosphate, we also measured alkaline phosphatase activity (Figure 6a).

Strains with a wild-type background (Figure 6a, datasets 1 and 6) showed the expected high AP activity in P-starved cells and low AP activity in cells grown with excess Pi (2 mm). As expected, the various *phoUB* and *sfx2* mutant strains contained minimal AP activities regardless of the level of Pi in the growth medium.

The level of *orfA-pit* expression (β -galactosidase activ-

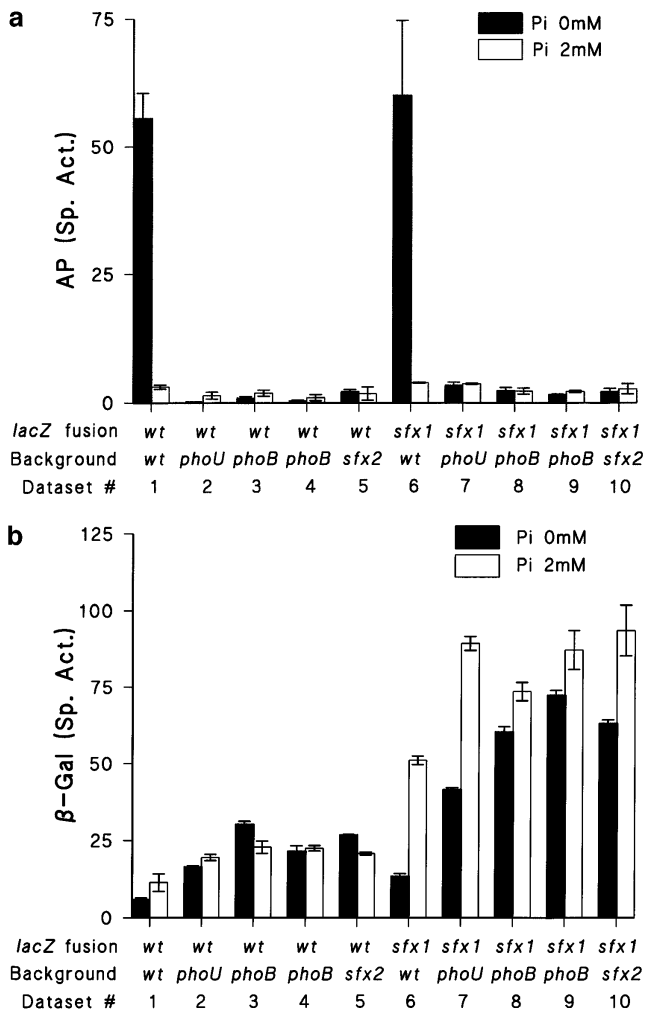


Figure 6.—Alkaline phosphatase- (a) and β -galactosidase- (b) specific activities of an *orfA-pit::lacZ* chromosomal fusion in wild type (wt), *phoU10* (#2 and 7), *phoB8* (#3 and 8), *phoB3* (#4 and 9) and *sfx2* backgrounds. *pit* expression from both wild-type and *sfx1* loci were tested in these backgrounds. The assay was performed after 32 hr growth in MOPS minimal medium with no phosphate added (MOPS P0, solid box) or in a medium supplemented with 2 mM Pi (MOPS P2, open box). Each value corresponds to the mean of triplicate assays \pm SE. Datasets are as follows: (1) RmH662, (2) RmH754, (3) RmH755, (4) RmH756, (5) RmH765, (6) RmH771, (7) RmH861, (8) RmH862, (9) RmH863, and (10) RmH860. β -galactosidase activities are expressed as Miller Units and AP specific activities were determined as described in materials and methods.

ity) directed from the *sfx1* promoter was higher than the level directed from the wild-type *orfA-pit* promoter (Figure 6b, datasets 1–5 vs. 6–10); and in the wild-type background, both wild-type and *sfx1*-directed *orfA-pit* expression was phosphate-regulated as the β -galactosidase activity was two- to fourfold higher in cells grown with 2 mM phosphate compared to phosphate-starved cells (Figure 6b, datasets 1 and 6). In the *phoU*, *phoB*, and *sfx2* backgrounds, *orfA-pit* expression increased 4.5–5.5-fold when the cells were cultured in the absence of added Pi, and 1.5–2-fold when the cells were grown with

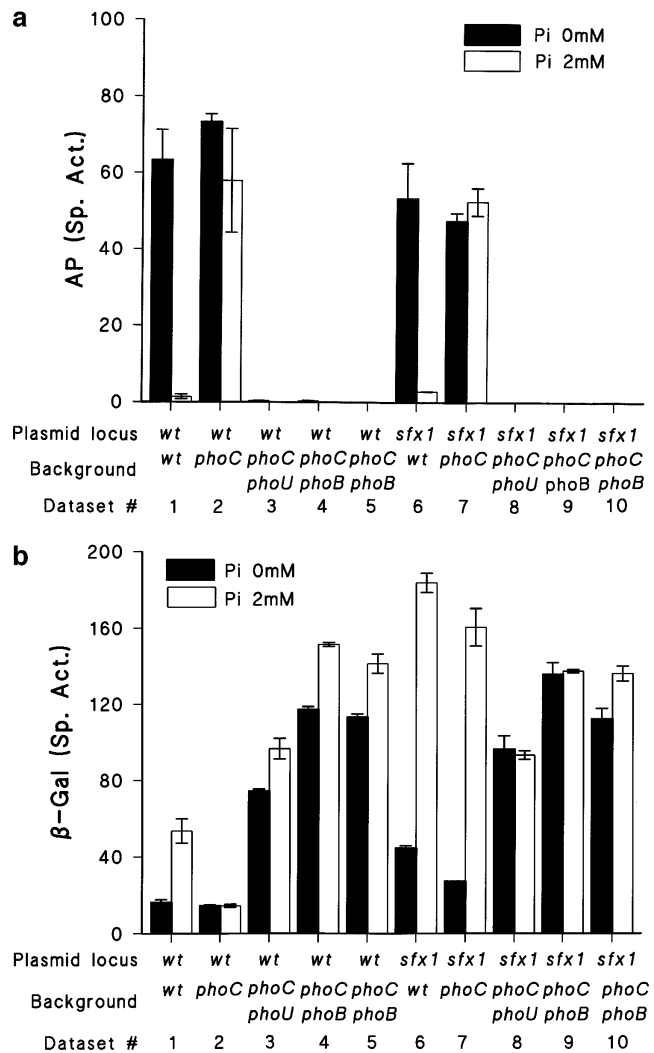


Figure 7.—Alkaline phosphatase (a) and β -galactosidase (b) activities of a plasmid-born *pit::lacZ* fusion in wild type (wt), *phoC* Ω 490 (*phoC*), *phoU10 phoC*, *phoB8 phoC* (datasets 4 and 9), *phoB3 phoC* (datasets 5 and 10), and *sfx2 phoC* backgrounds. The assays were performed after 38 hr growth in MOPS-buffered minimal medium with no phosphate added (MOPS P0, solid box) or in medium supplemented with 2 mM Pi (MOPS P2, open box). Each value corresponds to the mean of triplicate values \pm SE. β -galactosidase activities are expressed as Miller Units and AP specific activities were determined as described in materials and methods.

excess Pi (2 mM) relative to *orfA-pit* expression in a wild-type background (Figure 6b, datasets 1 vs. 2–5, and 6 vs. 7–10). These data suggest that *phoB* (*phoU*) is negatively regulating *orfA-pit* expression. Moreover, in the *phoB* and *sfx2* backgrounds the phosphate-dependent regulation of *orfA-pit* expression was dramatically reduced, suggesting that phosphate regulation of *orfA-pit* expression was probably mediated via PhoB. The results outlined above were obtained from the chromosomal *orfA-pit::lacZ* gene fusions. We obtained similar data when *pit* expression from a plasmid-borne gene fusion was assayed in these strains (data not shown).

As *phoB* and *phoU* mutations suppressed the Fix- and

phosphate-growth phenotype of *phoCDET* mutants, we wished to examine the influence of the *phoU*, *phoB*, and *sfx2* mutations on the level of *orfA-pit* expression observed in a *phoCDET* mutant background. In these experiments, expression from plasmid-borne wild-type and *sfx1*-directed *orfA-pit::lacZ* fusions in *phoC phoB* and *phoC phoU* backgrounds was monitored, and again AP activities were measured as controls (Figure 7, a and b). We note that *phoC* mutant cells behave as if they are phosphate-starved, even in the presence of excess phosphate (Figure 7a, dataset 2; Figure 1). In the *phoC* background, under both culture conditions, *orfA-pit* expression directed from the wild-type promoter was as low as its expression in phosphate-starved wild-type cells (Figure 7b, datasets 1 and 2). The introduction of a *phoB* or *phoU* mutation resulted in a greater than fivefold increase in the level of *orfA-pit* expression, suggesting that the repression of *orfA-pit* expression in *phoC* cells was mediated by PhoB or PhoU (Figure 7b, dataset 2 vs. 3–5). In the case of *sfx1*-directed *orfA-pit* expression, the level of expression observed in the *phoC phoB* and *phoC phoU* double mutants was two- to fourfold higher than the basal level observed in phosphate-starved wild-type cells and in *phoC* cells (Figure 7b, datasets 8–10 vs. 6 and 7). Thus, under phosphate starvation conditions, PhoB and/or PhoU appear to repress *sfx1*-directed *orfA-pit* expression. In cells cultured in 2 mM Pi, the level of *sfx1*-directed *orfA-pit* transcription was slightly higher in the wild-type and *phoC* background than in the *phoC phoB* and *phoC phoU* double mutants (Figure 7, datasets 6 and 7 vs. 8–10). Thus, the combination of *sfx1* and *phoB* or *phoU* does not have an additive effect on the level of *pit* transcription. Lastly, we note that in a *phoC* background, *sfx1*-directed *orfA-pit* expression, but not AP synthesis, was responsive to the media phosphate concentrations (Figure 7, a and b, dataset 7). This difference between wild-type vs. *sfx1*-directed *orfA-pit* expression was previously observed (S. D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results).

DISCUSSION

Defined *phoB* and *phoU* mutants of *R. meliloti* were isolated and used to examine the role of *phoB* and *phoU* in the expression of the *phoCDET* and *orfA-pit* genes. Expression of both *phoD::lacZ* and *phoE::lacZ* gene fusions was completely abolished in *phoB* and *phoU* mutant strains (Figure 4). We showed earlier that *phoCDET* expression is strongly activated in response to Pi starvation, and had noted that two appropriately positioned PhoB-like binding sites are located in the *phoC* promoter (Bardin *et al.* 1996). Collectively, these data represent strong evidence that PhoB is a positive regulator of *phoCDET* transcription.

Our data also suggest that *phoB* plays an important role in determining the level of *orfA-pit* transcription (Figure 6b and Figure 7). In Pi-starved cultures, transcription of *pit* and *orfA* was clearly higher in the *phoB*

and *phoU* mutants than in the wild type (Figure 6b, dataset 1 vs. 2–5, data for *orfA* not shown). In addition, while the wild-type *pit* fusions showed increased expression in Pi-sufficient compared to Pi-deficient cells, no such Pi regulation was observed when *phoB* or *phoU* mutants were examined (Figure 6b, dataset 1 vs. 2–4). While we assume that PhoB is directly interacting with the *orfA-pit* promoter, our evidence is indirect.

We found that *phoB*, *phoU*, and *sfx2* mutants, which are phenotypically *phoCDET*⁻ (Figure 4 and data not shown), formed wild-type Fix⁺ nodules on alfalfa and grew like wild type in media containing 2 mM Pi (Table 2; Figure 5). This indicated that an alternative Pi transport system was induced in these mutants. Our data on the expression of *pit::lacZ* fusions in the *phoB* and *phoU* mutants (Figure 6b) also strongly suggest that the alternate uptake system is encoded by the *orfA-pit* locus. We recently demonstrated that the kinetics and specificity of Pi uptake in *phoB* mutant cells and in *phoC sfx1* mutant cells is very similar (Voegelé *et al.* 1997). As *phoC sfx1* double mutant cells appear to transport Pi via the OrfA-Pit system (S. D. Bardin, R. Voegelé, N. Falcioni and T.M. Finan, unpublished results), the transport data referred to above are consistent with the suggestion that the OrfA-Pit transport system is employed for Pi uptake in *phoB* or *phoU* mutant cells.

To account for the reciprocal pattern of *orfA-pit* and *phoCDET* expression observed in wild-type cells cultured in the presence and absence of Pi, we suggest that when the concentration of Pi is high (2 mM), PhoB is inactive and *orfA-pit* is derepressed (expressed), and the *phoCDET* system is not expressed. Conversely, under conditions of phosphate starvation, PhoB is activated, *orfA-pit* expression is repressed, whereas *phoCDET* expression is activated by PhoB. The pattern of *phoCDET* and *orfA-pit* expression reflects different physiological characteristics of the two transport systems. Thus, the OrfA-Pit system is a low-affinity Pi transporter and the PhoCDET system has a high-affinity for Pi (Voegelé *et al.* 1997).

When *R. meliloti phoC* insertion and *phoCDET* deletion mutants are cultured in media containing 2 mM Pi, the cells contain high AP activity and thus physiologically behave as though they are starved for phosphate (Figure 7a, dataset 2, data not shown). Under these conditions, the *orfA-pit* genes are repressed and the cell is then phenotypically both *orfA-pit* and *phoCDET* negative. The poor growth of *phoCDET* mutants in media containing 2 mM Pi therefore indirectly results from the lack of *orfA-pit* expression; and wild-type growth is restored via mutations which increase *orfA-pit* expression, such as *sfx1*, *phoB* or *phoU*.

Why *phoCDET* mutations lead to reduced *orfA-pit* expression is not clear. In *E. coli*, mutations in the PstSCAB transport system generate a Pho constitutive phenotype, which is not unlike that observed for *phoCDET* mutants of *R. meliloti*. The PstSCAB system is thus believed to be part of the Pi sensory system (Wanner 1996). If the PhoCDET system has a sensory role in *R. meliloti*, we

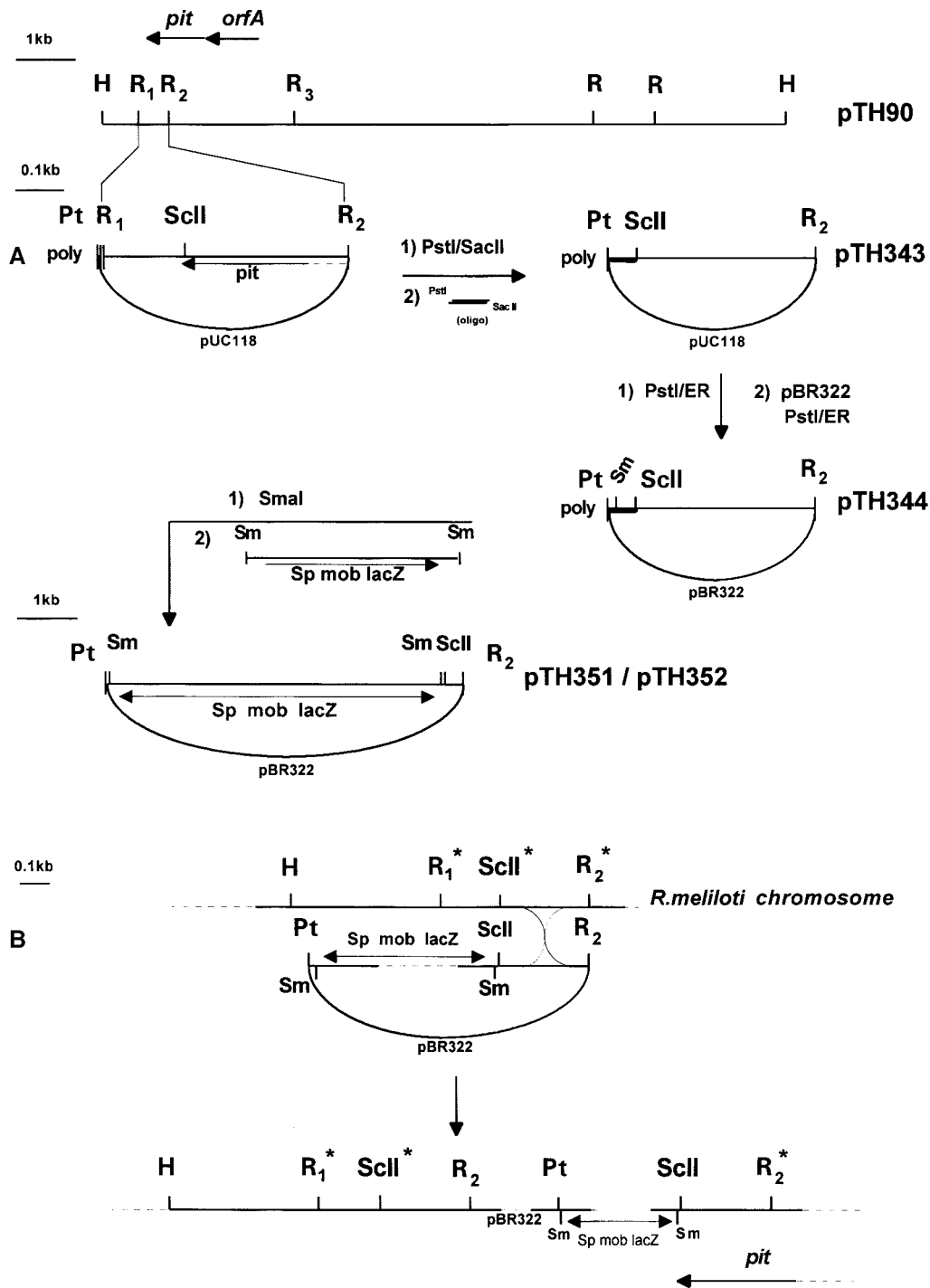


Figure 8.—Construction of the chromosomal *lacZ* fusions to *pit*. (A) The 0.5-kb *EcoRI* fragment (R1-R2) from pTH90 (containing the 3'-end of *stx1-pit*) was subcloned into pUC118 so that R1 was on the polylinker side. The subclone was digested with *SacI* (located 11 nucleotides upstream from the *pit* translational stop codon) and *PstI* (located in the polylinker), and the fragment was replaced by a linker (with *SacI/PstI* protruding ends) to create pTH343. The linker was constructed by hybridizing two complementary oligos (5'-GGACCTCGTCGCCTGACCCGGGCTGCA-3' and 5'-GCCCCGGGTCAGGCGACGA GGTCCGC-3') were synthesized so that the end of the *pit* gene was reconstituted (in order to obtain a functional gene). The translational stop codon was followed by a *SmaI* site used to clone the *lacZ* cassette. pTH343 was then digested with *PstI/EcoRI* and the fragment was cloned in pBR322 digested with the same enzymes to create pTH344. The *lacZ* cassette of the pLMS clone (pUC18 with the *Sp^r mob lacZ* cassette, M. Hynes, unpublished results) was subcloned as a *SmaI* fragment into pTH344 to create pTH351 and pTH352 with the *lacZ* gene in the same and opposite orientation as *pit*, respectively. (B) Following conjugative transfer from *E. coli* into *R. meliloti*, the plasmids recombined into the chromosome of a wild type and a *stx1* strain by single crossover homologous recombination in the 0.3kb *SacI/EcoRI*(2) fragment. Symbols used: poly: polylinker; H: *HindIII*; Pt: *PstI*; R: *EcoRI*; ScII: *SacII*; Sm: *SmaI*.

believe there must be at least another backup sensory system, as when phosphorus in the form of 2 mM AEP is supplied to *R. meliloti phoC* mutants, AP activity is completely repressed, and hence a phosphorous sensory system appears to function (Figure 1). Moreover, *E. coli pstSCAB* mutants, unlike *R. meliloti phoCDET* mutants, are reported to grow normally in media containing 1 mM Pi (Willsky and Malamy 1980). Phosphate transport in these *pstSCAB* mutants is believed to occur via a constitutive Pit phosphate transport system (Rosenberg 1987).

We draw attention to the fact that the *phoB* gene is located 111 bp downstream from *phoU* in *R. meliloti* (GenBank accession no. M96261). As both of these genes are transcribed in the same direction, it is probable that *phoU* and *phoB* are transcribed as a single mRNA, in which case *phoU* insertion mutants would also be phenotypically *PhoB*⁻. If this is the case, elucidation of the role of *phoU* in *R. meliloti* will require the construction of defined *phoU*⁻ *phoB*⁺ strains. The low Pi-regulated expression of *phoD* and *phoE* observed in a *phoU10* mutant background (Figure 4, dataset 2), together with the apparent Pi-dependent regulation of *sfx1*-directed *orfA-pit* expression also observed in the *phoU10* background, (Figure 6, dataset 7) suggest that the *phoU10::TnV* insertion may allow a low level transcription of *phoB*.

The AP⁻ phenotype of the *R. meliloti phoB* mutants is similar to that observed for *phoB* mutants in other bacteria (Lee *et al.* 1989; Anba *et al.* 1990). On the other hand, the AP⁻ phenotype of the *phoU* mutant is unusual as *phoU* mutants of both *E. coli* and *Pseudomonas aeruginosa* are constitutive for AP activity (Steed and Wanner 1993; Kato *et al.* 1994). *E. coli phoU* mutants grow poorly in MOPS media containing 2 mM Pi (Steed and Wanner 1993). While we have not seen any analogous phenotype with the *R. meliloti phoU* insertion mutant, we again note that it is very probable that these mutants are also *phoB*⁻.

Our screening experiment did not result in the isolation of any *phoR* mutants. It is possible that in the absence of PhoR other kinases cross-activate PhoB as observed in *E. coli* (Wanner and Wilmes-Riesenberg 1992; Wanner 1992).

Most studies of the Pho regulon have focused on genes whose expression increases in response to Pi limitation. However, over 20 years ago, Willsky and Malamy (1976) showed that three *E. coli* periplasmic proteins were only synthesized during growth in excess phosphate medium and not during growth in phosphate-limited medium. Moreover, synthesis of two of these proteins was clearly derepressed in a *phoB* mutant background. More recently, VanBogelen *et al.* (1996) estimated that the synthesis rate of 413 proteins in *E. coli* was modified under phosphate limitation, of which 208 were induced and 205 repressed. They noted that the promoter regions of three repressed genes, *ompF*, *pfl*, and *ssb* do contain putative PhoB boxes. In addition, Smith and Payne (1992) have suggested that PhoB may

repress expression of periplasmic peptide transport-binding proteins under low phosphate conditions. They identified a putative PhoB box that seems to overlap with the RNA polymerase-binding site.

sfx1 and *sfx2* were originally identified as mutations which suppressed the symbiotic Fix⁻ phenotype of *phoCDET(ndvF)* mutants (Oresnik *et al.* 1994). As *sfx1*, *sfx2*, increase *orfA-pit* expression and presumably OrfA-Pit-mediated Pi transport (S. D. Bardin, R. Voegelé, N. Falcioni and T. M. Finan, unpublished results; and this work, data not shown), we conclude that the symbiotic phenotype of *phoCDET (ndvF)* mutants is a direct consequence of their inability to transport sufficient Pi for cellular growth.

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