Characterization of the *Rhizobium* (*Sinorhizobium*) *meliloti* High- and Low-Affinity Phosphate Uptake Systems

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**Genetic studies have suggested that *Rhizobium* (*Sinorhizobium*) *meliloti* contains two distinct phosphate (Pᵢ) transport systems, encoded by the *phoCDET* genes and the *orfA-pit* genes, respectively. Here we present data which show that the ABC-type PhoCDET system has a high affinity for Pᵢ (Kₘ, 0.2 µM) and that Pᵢ uptake by this system is severely inhibited by phosphonates. This high-affinity uptake system was induced under Pᵢ-limiting conditions and was repressed in the presence of excess Pᵢ. Uptake via the OrfA-Pit system was examined in (i) a *phoC* mutant which showed increased expression of the *orfA-pit* genes as a result of a promoter-up mutation and (ii) a *phoB* mutant (*PhoB* is required for *phoCDET* expression). Pᵢ uptake in both strains exhibited saturation kinetics (Kₘ, 1 to 2 µM) and was not inhibited by phosphonates. This uptake system was active in wild-type cells grown with excess Pᵢ and appeared to be repressed when the cells were starved for Pᵢ. Thus, our biochemical data show that the OrfA-Pit and PhoCDET uptake systems are differentially expressed depending on the state of the cell with respect to phosphate availability.**

Phosphorus is an essential nutrient, and cells satisfy their demand for this element by uptake of inorganic phosphate (Pᵢ) and organic phosphate compounds. In most soils, soluble phosphate is present at 0.1 to 10 µM (6). These low concentrations result from the formation of essentially insoluble metallophosphate compounds which are produced upon addition of Pᵢ to soil.

The soil bacterium *Rhizobium* (*Sinorhizobium*) *meliloti* forms N₂-fixing root nodules on alfalfa. Our interest in phosphorus metabolism arose through analysis of the symbiotic *ndvF* locus, which is located on the 1,700-kb megaplasmid of this bacterium (8, 9). *R. meliloti* *ndvF* mutants form nodules which contain few bacteria and fail to fix N₂ (*Fix²*). Nodules which fix N₂ (*Fix¹*) were occasionally observed to form on plants inoculated with *ndvF* mutants, and genetic analysis showed that these nodules contained bacteria carrying one of two classes of second-site mutations which suppressed the *ndvF Fix²* phenotype (17). The *ndvF* locus was recently shown to consist of the *phoCDET* genes, which together encode an ABC-type transport system for the uptake of phosphate in *R. meliloti*. The *ndvF* locus was therefore redesignated *phoCDET* (4).

In addition to their *Fix²* phenotype, *phoCDET* mutants grew slowly in medium containing 2 mM Pᵢ as the sole source of phosphate. Phosphate uptake in Pᵢ-starved *phoCDET* mutant cells was less than 10% of that in wild-type cells when assayed at an external Pᵢ concentration of 10 µM (4). While the latter results suggested that PhoCDET was the sole phosphate uptake system in *R. meliloti*, recent analyses of the strains carrying either of the two classes of *phoCDET* (*ndvF*) second-site mutations, referred to above, indicated that an additional Pᵢ transport system is present (2, 3). The results of these studies showed that both classes of mutations lead to elevated expression of the *orfA-pit* operon. The *pit*-encoded protein showed homology to the *Escherichia coli* phosphate transport protein, Pit, and other Pᵢ transporters from various eukaryotic and prokaryotic organisms (3). One suppressor mutation, *sfx1*, was identified as containing a single-base deletion in the putative *orfA-pit* promoter region. Other suppressor mutations contained insertions in the *phoU* *phoB* locus, suggesting that *PhoB* negatively regulates *orfA-pit* expression (2, 3).

In *Escherichia coli*, two phosphate transport systems, Pit and *PstSCAB*, have been characterized. The Pit system is believed to be constitutively expressed, and it is described as a metallophosphate/proton symporter (22, 23, 25). This system exhibits a lower affinity and a higher Vₘₐₓ than the high-affinity ABC-type *PstSCAB* system. Expression of the *pstSCAB* genes is activated upon Pᵢ starvation, and this response is regulated by the two-component sensor regulator system, PhoR and PhoB (19, 23).

Here we report on the biochemical characterization of the PhoCDET and the Pit phosphate transport systems of *R. meliloti*. We demonstrate that the two systems can be distinguished on the basis of their affinity for phosphate and their response to inhibition by phosphonates. The picture evolving from this analysis suggests that phosphate uptake in *R. meliloti* is due to at least two phosphate uptake systems which are differentially expressed under different growth conditions.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Cells were grown in Luria-Bertani (LB) medium (16) supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc medium) or in MOPS minimal medium with or without 2 mM KH₂PO₄ (4). Minimal medium was supplemented at 15 µliter with a yeast extract fraction which stimulates the growth of *R. meliloti* in defined medium (24). Glucose and succinate were added to final concentrations of 15 mM each as carbon sources and for induction of the *acr* system. All the strains used in this study were derivatives of *R. meliloti* Rm1021 (14).

**Transport assays.** For transport assays, cells were precultured in LBmc medium, washed three times with MOPS-I (40 mM morpholinopropane sulfonic acid [MOPS], 20 mM KOH, 20 mM NH₄Cl, 100 mM NaCl, 2 mM MgSO₄·7H₂O, 1.2 mM CaCl₂, 0.5 µg of biotin per ml, 15 mM glucose), and subcultured into MOPS minimal medium with or without phosphate. Cells grown aerobically for 24 h at 30°C were harvested by centrifugation, washed four times with MOPS-I, and resuspended to an optical density at 600 nm (OD₆₀₀) of 10 in MOPS-I. Cells were diluted 1:20 into MOPS-I and equilibrated for 5 min at 30°C. Uptake was initiated by the addition of [³²P]orthophosphoric acid (DuPont, NEN Research Products, Boston, Mass.). Aliquots were removed from the assay at different time points, placed on nitrocellulose filters (pore size, 0.45 µm; HAWP 025 00; Millipore, Bedford, Mass.) presoaked in 1 M K₂HPO₄/KH₂PO₄, (pH 7.0), and immediately washed with 10 ml of MOPS-II (40 mM MOPS, 20 mM KOH, 20

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mM NH₄Cl, 100 mM NaCl, 1.2 mM CaCl₂, 2 mM MgSO₄. The filters were dried, placed in scintillation liquid (BCS; Amersham, Little Chalfont, England), and counted. Depending on the phosphate concentration, the specific activity of ³²P in the assay ranged from 60 to 704 Ci/mol. Inhibition experiments were done under standard assay conditions, except that inhibitors were added to a final concentration of 5 or 50 µM 4 min after the cells were added to the assay buffer for preincubation. ³²P (60 to 176 Ci/mol) was added 1 min later to a final concentration of 1 µM for phosphate-starved cells and 4 µM for cells grown in medium supplemented with 2 mM phosphate. Succinate uptake assays were performed as control experiments for all strains. [¹⁴C]succinate (DuPont, NEN) was used at a final concentration of 40 µM (2.5 to 4 Ci/mol). All the transport assays were performed in triplicate, and the values reported represent the mean ± standard error. Uptake rates for all assays were linear over the 5-min period monitored, and all primary graphs extrapolated to zero within the experimental error. The kinetic parameters were analyzed by linear regression with the linear transformation (Hanes-Woolf plots) of the Michaelis-Menten equation.

**Growth experiments.** Cells were precultured in LBmc medium, washed three times with MOPS-I, and diluted into MOPS-I (no phosphate added). The cultures were grown aerobically for 24 h at 30°C, and the OD₆₀₀ was adjusted to 0.2. This cell suspension was diluted 1:1,000 into 5 ml of MOPS-I containing the indicated phosphorus source at 2 mM.

**AP assays.** Alkaline phosphatase (AP) assays were performed on all minimal medium cultures prior to transport experiments to determine the physiological status of the cells with respect to phosphate, as described by Yarosh et al. (26). The AP activity was calculated by the following formula: specific activity = (1,000 × OD₆₀₀)/(OD₆₀₀ × Δ).

**Protein determination.** Protein concentrations were determined by the Bradford method (7) with the Bio-Rad protein assay dye; dilutions of bovine serum albumin were used as the standard.

**RESULTS**

**Characterization of the PhoCDET system.** We have previously shown that *R. meliloti* wild-type cells (Rm1021) are grown under phosphate starvation, the *phoCDET* genes are induced and the cells take up phosphate when it is provided at 10 µM (4). It was further demonstrated that when a *phoCDET* mutant was grown and assayed under identical conditions, no significant Pᵢ accumulation occurred (4). To determine the kinetic parameters for the PhoCDET uptake system, Pᵢ uptake rates in phosphate-starved cells of the wild-type strain Rm1021 were measured over a substrate concentration range between 0.05 and 3 µM. Michaelis-Menten plots of the resulting data indicated a classical hyperbolic behavior of the PhoCDET system, and the *Kₚᵢ* and *Vₘₐₓ* values determined from Hanes-Woolf plots were found to be 0.2 µM and 6.8 nmol/min/mg of protein, respectively (Fig. 1). The low *Kₚᵢ* value supports the classification of PhoCDET as a member of the high-affinity, binding protein-dependent phosphate uptake systems similar to the PstSCAB system from *E. coli*.

The homology of *R. meliloti* *phoCDET* genes to the *E. coli* *phnCDE* phosphonate uptake genes, together with results from growth experiments (4), suggested that PhoCDET might transport phosphonates in addition to phosphate. To investigate this possibility in more detail, and since no suitable radiolabeled phosphonates were commercially available for direct uptake experiments, we tested the ability of several phosphonates to inhibit phosphate uptake at concentrations 5- and 50-fold above that of phosphate (Fig. 2). The addition of unlabeled Pᵢ showed the reduction in the uptake of the labeled substrate one would expect from simple dilution of the label with unlabeled Pᵢ. All of the phosphonates except aminomethylphosphonate were potent inhibitors of Pᵢ uptake. On the other hand, arsenate, which is a structural analog of phosphate (5), inhibited uptake to a degree similar to the effect observed by dilution of the label with unlabeled Pᵢ. At the higher inhibitor concentration, the inhibition of phosphate uptake was almost identical for ethylphosphonate, methylphosphonate, arsenate, and excess Pᵢ. If we assume competitive inhibition, these results indicate that the PhoCDET system exhibits a broad substrate specificity covering phosphonates as well as Pᵢ.

Genetic analysis of two classes of second-site mutations which suppress the symbiotic Fix⁻ phenotype of *phoCDET*
with 2 mM phosphate are shown. (Insert) Hanes-Woolf plots to identify $K_m$.

Labeled $P_i$ was added to 4 ($490 sfx1$) cells grown in medium supplemented with 2 mM phosphonates and arsenate was determined with RmG762 ($490 sfx1$) ($490 sfx1$) grown in medium supplemented with 2 mM phosphonates. The class I suppressor mutation, $sfx1$, appeared as a promoter mutation which directly increases the expression of the orfA-pit genes. The class II suppressor mutations mapped to the $phoU$ and $phoB$ regulatory genes and also increased orfA-pit expression. Below, we characterize the kinetics and specificity of phosphate transport in these suppressor strains.

**Characterization of $sfx1$-dependent phosphate uptake.**

The kinetics and the substrate specificity of phosphate uptake in the suppressor mutant RmG762 ($phoCO490 sfx1$) (3) were determined with cells grown in medium containing 2 mM phosphate. The uptake rates were measured over a substrate concentration range between 1 and 32 $\mu$M, and Michaelis-Menten plots of the resulting data indicated a classic hyperbolic curve typical of a saturable carrier-mediated transport system, and $K_m$ and $V_{max}$ values of 2.1 $\mu$M and 1.4 nmol/min/mg of protein, respectively, were derived from Hanes-Woolf plots (Fig. 3).

![FIG. 3. The Pit system exhibits different kinetics from the PhoCDET system. Michaelis-Menten plots for phosphate uptake in strain RmG762 ($phoCO490 sfx1$) (■) and strain RmH838 ($phoB3::Tn5$) (●) grown in medium supplemented with 2 mM phosphate are shown. (Insert) Hanes-Woolf plots to identify $K_m$ and $V_{max}$. The kinetic parameters obtained from the Hanes-Woolf plots were used in the Michaelis-Menten equation to produce the fit for the data in the Michaelis-Menten plot. AP and succinate uptake values for this experiment were 1.4 U and 31.4 ± 1.8 nmol/min/mg of protein for strain RmG762 and 0.2 U and 52.0 ± 1.8 nmol/min/mg of protein for strain RmH838, respectively.](http://jb.asm.org/)

FIG. 4. $P_i$ uptake via the Pit system is not inhibited by phosphonates. The bar graph depicts the inhibition of phosphate uptake in strain RmG762 ($phoCO490 sfx1$) □ and in strain RmH838 ($phoB3::Tn5$) by various inhibitors. Inhibition experiments were carried out at a phosphate concentration of 4 $\mu$M and an inhibitor concentration of 50 $\mu$M with cells grown in medium containing 2 mM phosphate. The data are presented as the percent residual activity compared to the control. The rates of the controls (no inhibitor) were 0.4 nmol/min/mg of protein for strain RmG762 and 1.0 nmol/min/mg of protein for strain RmH838. Abbreviations: control, no addition; EP, ethylphosphonate; AEP, aminomethylphosphonate; MP, methylphosphonate; AMP, aminomethylphosphonate; asn, arsenate. AP and succinate uptake values for this experiment were 1.4 U and 31.4 ± 1.7 nmol/min/mg of protein for strain RmG762 and 0.2 U and 52.0 ± 1.8 nmol/min/mg of protein for strain RmH838, respectively.

mutants suggested that a second phosphate transport system, in addition to the PhoCDET system, was operative in R. meliloti (2, 3). The suppressor mutations allowed phoCDET mutants to grow like the wild type in medium supplemented with 2 mM phosphate. The class I suppressor mutation, $sfx1$, appears to be a promoter mutation which directly increases the expression of the orfA-pit genes. The class II suppressor mutations mapped to the $phoU$ and $phoB$ regulatory genes and also increased orfA-pit expression. Below, we characterize the kinetics and specificity of phosphate transport in these suppressor strains.

Phosphate uptake in a $phoB$ mutant. Expression of phoCDET is positively regulated by PhoB, and hence $phoB$ insertion mutants are phenotypically PhoCDET− (2). However, $phoB$ insertion mutations suppress the symbiotic Fix− phenotype and the slow growth of phoC mutants on medium containing 2 mM $P_i$ (2). To further investigate the phenotypic effects of the $phoB$ and phoC mutations, we examined the ability of the wild-type strain Rm1021, the $phoC$ mutant RmG490 ($phoCO490$) (9), and the $phoB$ mutant RmH838 ($phoB3::Tn5$) (2) to grow in defined medium containing various sources of phosphorus at 2 mM. Unlike the $phoC$ mutant, the $phoB$ mutant was clearly defective in its ability to utilize methyl- and aminomethylphosphonate (Fig. 5). In contrast, whereas the $phoC$ mutant grew slowly in medium containing 2 mM $P_i$, the $phoB$ mutant grew like the wild type in this medium. Since previous gene expression studies indicated that suppression of the phoCDET mutations by $phoB$ resulted from increased expression of the orfA-pit operon (2), we wished to compare the kinetics and specificity of phosphate uptake in the $phoB$ mutant RmH838 ($phoB3::Tn5$) with the parameters determined for the $sfx1$ suppressor strain RmG762 ($phoCO490 sfx1$). RmH838 cells were grown in medium containing 2 mM phosphate, and the $P_i$ uptake rates were measured at substrate concentrations between 1 and 32 $\mu$M. Michaelis-Menten plots of the resulting data indicated a classic hyperbolic curve typical of a saturable carrier-mediated transport system, and $K_m$ and $V_{max}$ values of 2.1 $\mu$M and 1.4 nmol/min/mg of protein, respectively, were derived from Hanes-Woolf plots (Fig. 3).
The sensitivity of P$_i$ uptake in RmH838 (phoB3::TnV) to inhibition by phosphonates and arsenate was determined under identical conditions to those used for the sfx1 suppressor strain RmG762. The results mirrored those obtained for the sfx1 suppressor strain RmG762 (Fig. 4). Uptake was significantly inhibited by arsenate, but the phosphonates tested caused little if any inhibition. Dilution of the radiolabel with excess unlabeled P$_i$ resulted in the expected reduction of uptake of labeled P$_i$.

The virtually identical response to inhibitors and the similar $K_m$ values for P$_i$ uptake in the two classes of suppressor mutants (1.5 μM for the class I suppressor and 2.1 μM for the class II suppressor) suggest that phosphate is transported by the same system in both strains. Our results therefore support the proposal that suppression of phoCDET mutations by phoB mutations is due to increased expression of the orfA-pit operon (2, 3). For simplicity, we refer to the transport system observed in RmG762 (phoC490 sfx1) and RmH838 (phoB3::TnV) as the Pit transport system. However, we emphasize that we have not yet determined whether the orfA gene product is required for the observed P$_i$ transport. Having established the existence of this second P$_i$ uptake system, we wished to determine how much this uptake system contributes to the total phosphate uptake in wild-type cells.

Phosphate uptake in wild-type cells. Since the Pit system exhibited a higher $K_m$ and a lower $V_{max}$ than the PhoCDET system, it was not feasible to determine the kinetics of P$_i$ uptake via the Pit system in a wild-type background. Because of the differential responses of the PhoCDET and Pit uptake systems to inhibition by phosphonates, we examined the inhibition pattern of phosphate uptake in wild-type Rm1021 cells grown in medium containing 2 mM P$_i$. The results showed that P$_i$ uptake was strongly inhibited by arsenate, but the phosphonates tested caused little if any inhibition. Dilution of the radiolabel with excess unlabeled P$_i$ resulted in the expected reduction of uptake of labeled P$_i$.

The above inhibition data do not exclude the possibility that another P$_i$ uptake system with similar specificity to the Pit system operates in wild-type cell growing with 2 mM P$_i$. To investigate this possibility, we also examined the inhibition pattern of phosphate uptake in cells of the pit310::Tn5 insertion mutant, RmG804, following growth of the mutant in medium containing 2 mM phosphate.
cells grown in medium supplemented with 2 mM phosphate. The succinate uptake activity was assayed at a substrate concentration of 4 mM, we found that the rate of Pi uptake in the double mutant RmG830 (orfA-pit) was virtually identical to that observed for the PhoCDET system in phosphate-starved wild-type cells (Fig. 5B). The low Km value of about 1.8 μM for Pi uptake via the Pit-like transport system (the mean of the values for RmG762 [1.5 μM] and RmH838 [2.1 μM]), together with the insensitivity of Pi transport to inhibition by phosphonates, indicates that Pi is a low-affinity, high-specificity Pi uptake system. Thus, the R. meliloti Pit system has a much higher substrate specificity and lower substrate affinity than the PhoCDET transport system.

In E. coli, there are two major phosphate uptake systems, the above-mentioned PstSCAB system and the Pit system. The Pit system is described as a low-affinity, high-velocity proton motive force-driven transport system which probably transports metallophosphate in symport with a proton (Km, 38.2 μM; Vmax, 55 nmol/min/mg [dry weight]) (25). In E. coli, Pi uptake via the Pit system shows a higher velocity than the PstSCAB system, whereas in R. meliloti, the uptake rates measured for the Pit system were clearly lower than the rates measured for the PhoCDET system. In preliminary experiments, we examined the effect of altering the concentration of Mg2+ and Ca2+ in the assay mixtures. While our data do not allow us to distinguish whether uptake of metallophosphate is occurring, Pit-directed uptake was not stimulated by increasing the level of Ca2+ or Mg2+ in the assay mixture (data not shown).

The biochemical properties of the R. meliloti Pit uptake system are of particular interest given our recent study of orfA-pit and phoCDET expression in R. meliloti (2, 3). These studies led us to propose that under conditions of phosphate starvation, orfA-pit is repressed by PhoB whereas phoCDET expression is activated by PhoB. Conversely, under conditions of excess Pi (2 mM), orfA-pit is expressed and the OrfA-Pit transport system is can be explained by the presence of an uncharacterized uptake system(s) which is capable of transporting Pi at a very low uptake rate.

**DISCUSSION**

We have presented a biochemical characterization of two distinct phosphate uptake systems in R. meliloti. The two systems differ in their kinetic properties, their response to inhibition by phosphonates, and their genetic regulation. The transport system designations PhoCDET and Pit refer to the putative gene products responsible for Pi transport. The Pit protein is encoded by the orfA-pit operon, and the possible involvement of the OrfA protein in Pi transport has not yet been investigated (3).

The kinetic parameters determined for the PhoCDET system were a Km of 0.2 μM and a Vmax of 6.8 nmol/min/mg of protein. The low Km value is consistent with the genetic evidence that the PhoCDET uptake system is a member of a family of high-affinity ABC-type transporters (4, 10). The best-characterized phosphate transporter in this family is the PstSCAB system of E. coli, which has a Km of 0.4 μM and a Vmax of 15.9 nmol/min/mg (dry weight) (25). However, the R. meliloti PhoCDET proteins have sequence similarity to the E. coli PhnCDE proteins (4). The latter proteins constitute an ABC-type transporter specific for phosphonate, which is also capable of transporting phosphate (15). Our data showing that phosphonates inhibit phosphate uptake via PhoCDET suggest that the R. meliloti PhoCDET phosphate uptake system may also transport phosphonates. This view is further supported by the poor growth of the phoC mutant RmG490 (phoCDET) in medium containing ethylphosphonate as the sole source of phosphorus (Fig. 5B). However, the ability of the phoC mutant RmG490 to grow on other phosphonates demonstrates that phosphate uptake systems in addition to PhoCDET are present in R. meliloti (see below).

The Km value of about 1.8 μM for Pi uptake via the Pit-like transport system (the mean of the values for RmG762 [1.5 μM] and RmH838 [2.1 μM]), together with the insensitivity of Pi transport to inhibition by phosphonates, indicates that Pi is a low-affinity, high-specificity Pi uptake system. Thus, the R. meliloti Pit system has a much higher substrate specificity and lower substrate affinity than the PhoCDET transport system.

Slow growth of a phoCDET mutant on 2 mM phosphate. The biochemical and genetic data which clearly indicate the presence of two phosphate uptake systems in R. meliloti raise the question why phoCDET mutants grow slowly in medium containing 2 mM Pi, as the sole phosphorus source (Fig. 5B) (4). Since the Pit uptake system appears to be used exclusively for Pi uptake into wild-type R. meliloti cells growing in medium containing 2 mM Pi, we questioned whether this system was active in the phoCDET mutant strains. We therefore measured phosphate uptake in the wild-type Rm1021, the phoC mutant RmG490 (phoCDET), and the double mutant RmG830 (phoCDET pit) following growth of the cells in medium containing 2 mM phosphate (Fig. 7). With a substrate concentration of 4 mM, we found that the rate of Pi uptake in the phoC mutant cells was about 50% of that in the wild type. This result, together with the results from the growth experiments (Fig. 5), does suggest that mutations in the PhoCDET system reduce the expression and/or activity of the Pit system. However, the mechanism by which this regulation occurs remains to be elucidated. The phoC pit double mutant showed a further decrease in phosphate uptake, confirming that the uptake seen in the phoC mutant is indeed due to the Pit system. The very low rate observed with the phoC pit double mutant...
used for Pi transport, whereas the phoCDET genes are not expressed. We showed that in medium containing 2 mM Pi, phoCDET mutants behave as though they are phosphate starved and orfA-pit expression is very low. Both the sfx and phoB mutations were shown to increase orfA-pit transcription (2, 3). Our present data showing an identical substrate specificity of Pi uptake in strain RmG762 (phoCDET sfx1) and strain RmH838 (phoB::Tn5) (Fig. 4) suggest that the same transporter is being used in both of these strains. Further, the specificity of the Pi uptake system used in these strains is identical to the substrate specificity observed in wild-type cells grown in medium containing 2 mM Pi. Thus, the biochemical data are entirely consistent with the genetic data suggesting that the orfA-pit operon encodes a Pi transporter which is expressed in R. meliloti cells growing in the presence of excess Pi.

We have previously reported that whereas phoC mutants grow poorly in medium containing 2 mM Pi (4), phoC pit double mutants show a further decrease in their growth rate (3). In this study, we measured Pi uptake in the wild type, Rm1021, the phoC mutant RmG490 (phoCDET), and the double mutant RmG830 (phoCDET pit310::Tn5) (Fig. 7). The uptake rate of the phoC mutant cells was about 50% of that of the wild type, whereas the phoC pit double mutant showed a further reduction in uptake activity. These transport data are entirely consistent with the notion that the reduction in uptake rates in medium containing 2 mM Pi is a result of a reduction in Pi uptake as opposed to some other aspect of Pi assimilation. Since phoC mutants grown in 2 mM Pi are phytotypically phosphate starved, it is very likely that the signal transduction pathway regulating the expression of both Pi uptake systems in R. meliloti is disturbed by this mutation. This would explain the reduction in orfA-pit expression because of a false-negative signal (low Pi). We have not been able to determine the level of Pi activity in phosphate-starved wild-type cells; however, we believe that the activity of the Pi system determined in the phoC mutant grown in the presence of excess Pi (Fig. 7) does reflect the activity state of Pi under phosphate-starved conditions.

The ability of the phoC mutant to grow on phosphonates suggests that alternate uptake systems can transport these compounds. While the failure of the phoB mutant RmH838 (phoB::Tn5) to grow on phosphonates (except aminothio-ylphosphonate) might suggest that the alternate phosphate uptake system(s) is PhoB regulated, we note that it is also very likely that the genes encoding the C-P lyase enzyme complex are PhoB regulated in R. meliloti (13). The ability of the phoB mutant to grow on aminothioylphosphonate shows that this compound can be transported and/or degraded by a separate pathway from that for the other phosphonates examined. Pathways specific to the degradation of aminothioylphosphonate are known; however, in both Enterobacter aerogenes (12) and Salmonella typhimurium (11), the expression of these pathways is believed to be PhoB dependent.

In a previous study, Smart et al. (18) found that Pi-limited chemostat cultures of R. meliloti WU3 and Rhizobium sp. strain NGR234 took up Pi at least 10 times faster than phosphate-rich cultures did. The Km for Pi uptake in the Pi-limited cells was determined to be 6 and 4 μM, respectively. The authors concluded that a single, repressible phosphate uptake system was present in rhizobia. While we have also observed that the rate of Pi uptake in Pi-limited cells of R. meliloti Rm1021 is much higher than the uptake rate in Pi-sufficient cells, the Km determined for the Pi-limited cells (0.2 μM) is an order of magnitude lower than the values reported by Smart et al. (18). The differences between these results may reflect the different growth conditions or different bacterial strains used in the two studies. We note that we were initially concerned that the Pi uptake activities (0.6 to 1.4 nmol/min/mg of protein) measured for cells grown in medium containing 2 mM Pi were unusually low. However, in the above study by Smart et al. (18), the uptake rates for phosphate-rich cells of R. meliloti WU3 and Rhizobium sp. strain NGR234 were also low, at 1.3 and 0.4 nmol/min/mg (dry weight) of cells, respectively. Similar numbers were reported by Al-Niemi et al. (1) for Pi uptake in Pi-sufficient cells and bacteroids of Rhizobium tropici. Moreover, in many of the experiments, we observed that despite the apparent low rates of Pi transport, the cells showed the expected rates of succinate uptake (30 to 60 nmol/min/mg of protein), confirming that the cells were physiologically active.

Lastly, we note that the presence of two Pi uptake systems is conserved in microorganisms (20, 21). However, we are unaware of other instances where one of these systems is not homologous to the PstSCAB system of E. coli. In addition, it is perhaps worth noting that the sequence of the complete E. coli genome revealed the presence of two pit-like gene sequences. Whether both of these genes play a role in Pi transport in E. coli remains to be established.

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