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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 (P_i) 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 P_i (K_m, 0.2 μM) and that P_i uptake by this system is severely inhibited by phosphonates. This high-affinity uptake system was induced under P_i-limiting conditions and was repressed in the presence of excess P_i. 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). P_i uptake in both strains exhibited saturation kinetics (K_m, 1 to 2 μM) and was not inhibited by phosphonates. This uptake system was active in wild-type cells grown with excess P_i and appeared to be repressed when the cells were starved for P_i. 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 (P_i) 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 P_i to soil.

The soil bacterium *Rhizobium* (*Sinorhizobium*) *meliloti* forms N_2-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 N_2 (Fix^-). Nodules which fix N_2 (Fix^+) 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* Fix^- 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 Fix^- phenotype, *phoCDET* mutants grew slowly in medium containing 2 mM P_i as the sole source of phosphorus. Phosphate uptake in P_i-starved *phoCDET* mutant cells was less than 10% of that in wild-type cells when assayed at an external P_i 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 P_i 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 P_i 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 V_{max} than the high-affinity ABC-type PstSCAB system. Expression of the *pstSCAB* genes is activated upon P_i 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 $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc medium) or in MOPS minimal medium with or without 2 mM KH_2PO_4 (4). Minimal medium was supplemented at 15 μl/liter 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 *dct* 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 morpholinepropanesulfonic acid [MOPS], 20 mM KOH, 20 mM NH_4Cl, 100 mM NaCl, 2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.3 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 (OD_{600}) 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 [^{33}P]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 K_2HPO_4/KH_2PO_4 (pH 7.0), and immediately washed with 10 ml of MOPS-II (40 mM MOPS, 20 mM KOH, 20

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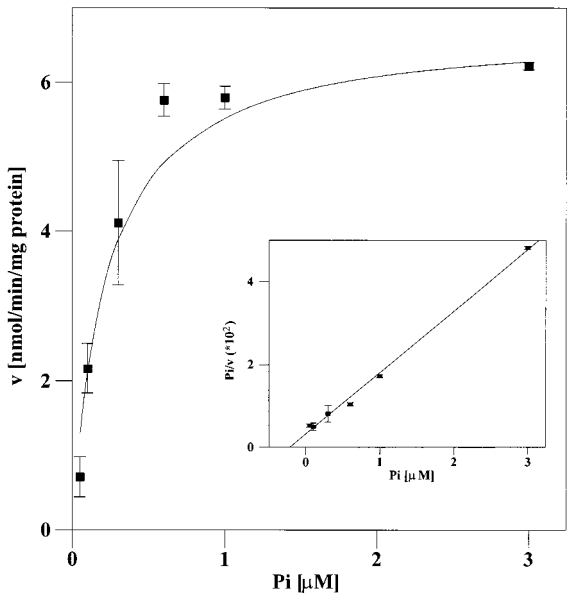

FIG. 1. The PhoCDET system is a high-affinity phosphate uptake system. A Michaelis-Menten plot for PhoCDET activity in cells of strain Rm1021 (wild type) grown in medium without phosphate is shown. (Insert) Hanes-Woolf plot to identify K_m and V_{max}. The kinetic parameters obtained from the Hanes-Woolf plot were used in the Michaelis-Menten equation to produce the fit for the data in the Michaelis-Menten plot. The AP value for cells used in this experiment was 35.6 U.

mM NH_4Cl, 100 mM NaCl, 1.2 mM $CaCl_2$, 2 mM $MgSO_4$). The filters were dried, placed in scintillation liquid (BCS; Amersham, Little Chalfont, England), and counted. Depending on the phosphate concentration, the specific activity of $^{33}P_i$ 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. $^{33}P_i$ (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. [^{14}C]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 OD_{600} 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 = $(1{,}000 \times OD_{420})/(OD_{600} \times \Delta t)$.

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 when *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 P_i accumulation occurred (4). To determine the kinetic parameters for the PhoCDET uptake system, P_i 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 K_m and V_{max} 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 K_m 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 P_i showed the reduction in the uptake of the labeled substrate one would expect from simple dilution of the label with unlabeled P_i. All of the phosphonates except aminomethylphosphonate were potent inhibitors of P_i 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 P_i. At the higher inhibitor concentration, the inhibition of phosphate uptake was almost identical for ethylphosphonate, methylphosphonate, arsenate, and excess P_i. If we assume competitive inhibition, these results indicate that the PhoCDET system exhibits a broad substrate specificity covering phosphonates as well as P_i.

Genetic analysis of two classes of second-site mutations which suppress the symbiotic Fix$^-$ phenotype of *phoCDET*

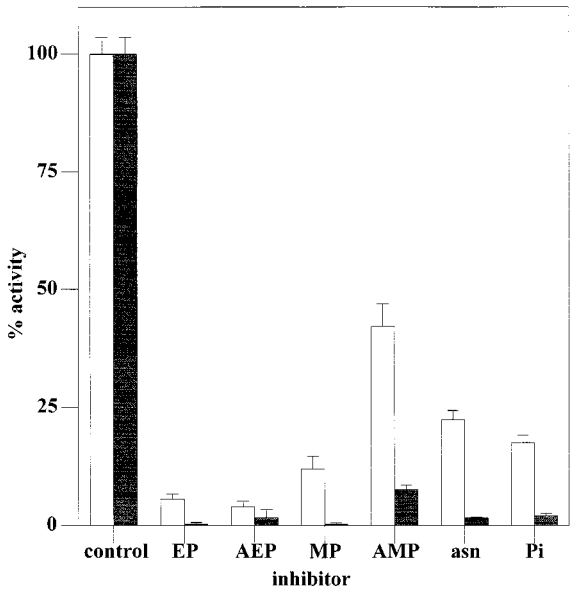

FIG. 2. The PhoCDET system is inhibited by phosphonates. The bar graph depicts the inhibition of phosphate uptake via PhoCDET by various inhibitors. Inhibition experiments with cells of Rm1021, grown in medium containing no phosphate, were carried out at a phosphate concentration of 1 μM and an inhibitor concentration of 5 μM ▭ or 50 μM ■. The data are presented as percent residual activity compared to the control. The rate of the control value (no inhibitor) was 5.5 nmol/min/mg of protein. Abbreviations: control, no addition; EP, ethylphosphonate; AEP, aminoethylphosphonate; MP, methylphosphonate; AMP, aminomethylphosphonate; asn, arsenate. The AP value for cells used in this experiment was 60.3 U.

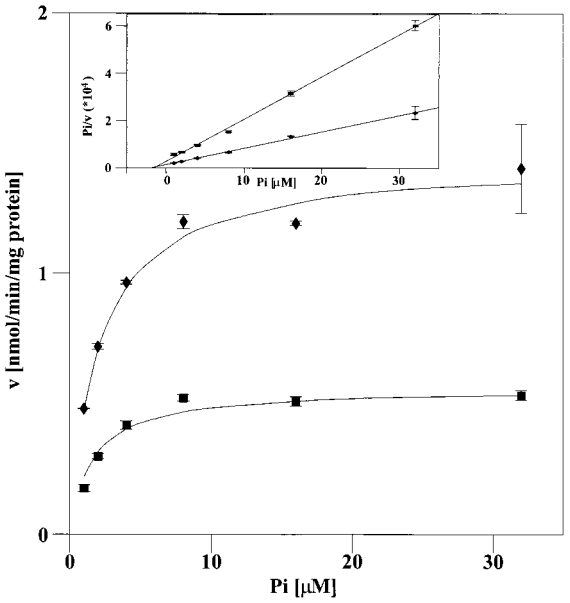

FIG. 3. The Pit system exhibits different kinetics from the PhoCDET system. Michaelis-Menten plots for phosphate uptake in strain RmG762 (*phoCΩ490 sfx1*) (■) and strain RmH838 (*phoB3*::Tn*V*) (◆) grown in medium supplemented with 2 mM phosphate are shown. (Insert) Hanes-Woolf plots to identify K_m 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 ± 1.7 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.

Characterization of *sfx1*-dependent phosphate uptake. The kinetics and the substrate specificity of phosphate uptake in the suppressor mutant RmG762 (*phoCΩ490 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 μM, and Michaelis-Menten plots of the resulting data indicated a classical hyperbolic behavior for P_i uptake over the concentration range tested. The K_m and V_{max} determined from Hanes-Woolf plots were 1.5 μM and 0.6 nmol/min/mg of protein, respectively (Fig. 3). Compared to the PhoCDET system observed in P_i-starved wild-type cells, P_i uptake in RmG762 showed a lower substrate affinity and a lower maximal rate.

The sensitivity of this P_i uptake system to inhibition by phosphonates and arsenate was determined with RmG762 (*phoCΩ490 sfx1*) cells grown in medium supplemented with 2 mM P_i. Labeled P_i was added to 4 μM, and inhibitors were tested at 50 μM (i.e., 12.5-fold excess). Arsenate significantly inhibited P_i uptake; however, the phosphonates tested showed little if any inhibition (Fig. 4). The addition of unlabeled P_i again led to the expected reduction in the uptake of labeled substrate. The lack of inhibition by phosphonates is in marked contrast to what was observed for P_i uptake in phosphate-starved wild-type cells.

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 (*phoCΩ490*) (9), and the *phoB* mutant RmH838 (*phoB3*::Tn*V*) (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*::Tn*V*) with the parameters determined for the *sfx1* suppressor strain RmG762 (*phoCΩ490 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 μM. 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 μM and 1.4 nmol/min/mg of protein, respectively, were derived from Hanes-Woolf plots (Fig. 3).

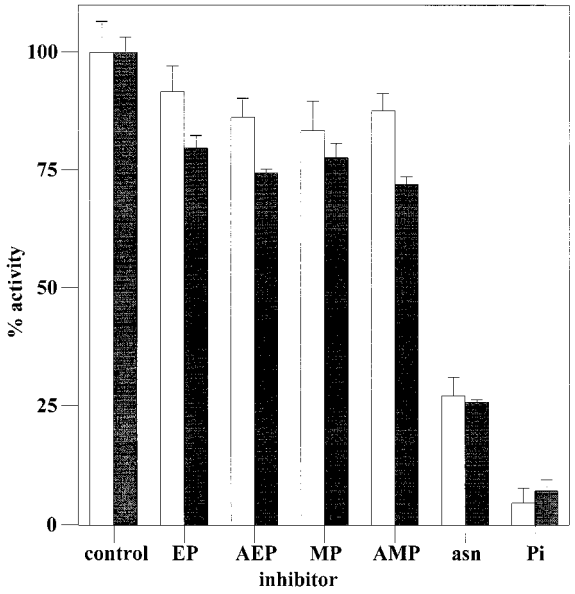

FIG. 4. P_i uptake via the Pit system is not inhibited by phosphonates. The bar graph depicts the inhibition of phosphate uptake in strain RmG762 (*phoCΩ490 sfx1*) ▭ and in strain RmH838 (*phoB3*::Tn*V*) ▬ by various inhibitors. Inhibition experiments were carried out at a phosphate concentration of 4 μM and an inhibitor concentration of 50 μM 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, aminoethylphosphonate; 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.

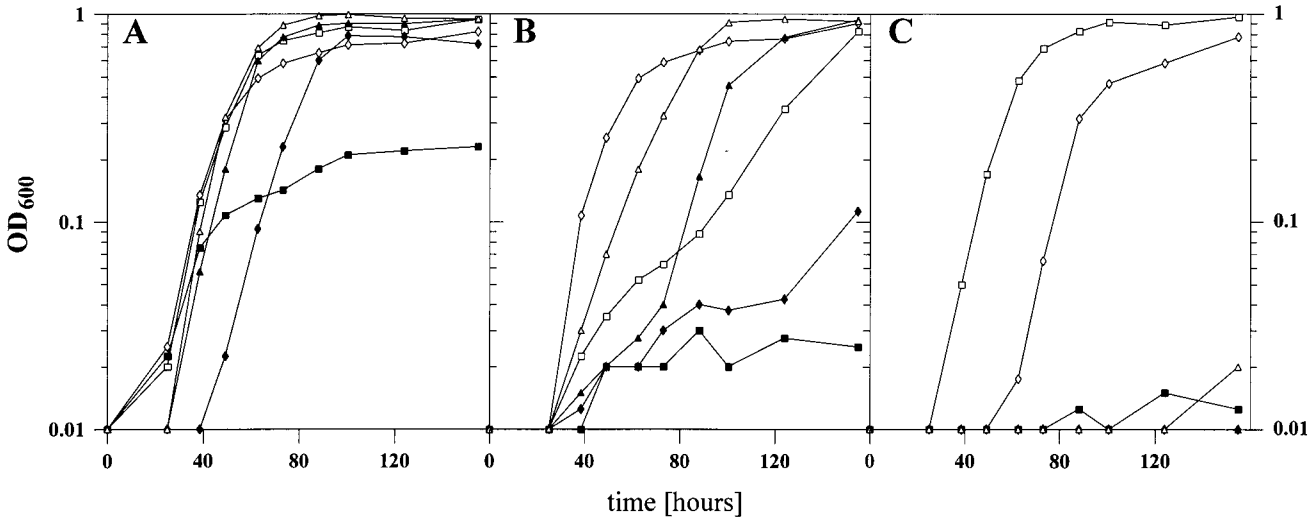

FIG. 5. Growth curves for the wild-type strain Rm1021 (A), the *phoC* mutant RmG490 (B), and the *phoB* mutant RmH838 (C) in MOPS minimal medium supplemented with glucose as the carbon source and different sources of phosphorus. The growth of the cultures was monitored by measuring the OD_{600}. The data represent the mean of triplicate experiments. Symbols; ■, no phosphorus source added; □, 2 mM phosphate; ◆, 2 mM ethylphosphonate; ◇, 2 mM aminoethylphosphonate; ▲, 2 mM methylphosphonate; △, 2 mM aminomethylphosphonate.

The sensitivity of P_i uptake in RmH838 (*phoB3*::Tn*V*) 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 (*phoCΩ490 sfx1*) and RmH838 (*phoB3*::Tn*V*) 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 *R. meliloti* 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 phosphate (Fig. 6). The results showed that P_i uptake was strongly inhibited by arsenate but was essentially unaffected by phosphonates. The response showed a pattern virtually identical to that observed for P_i uptake via the Pit system in strains RmG762 (*phoCΩ490 sfx1*) and RmH838 (*phoB3*::Tn*V*) (Fig. 4). Thus, the Pit uptake system appears to be used for P_i uptake in *R. meliloti* wild-type cells growing in 2 mM P_i.

Phosphate uptake in a *pit* mutant. 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*::Tn*5* insertion mutant, RmG804, following growth of the mutant in medium containing 2 mM phosphate

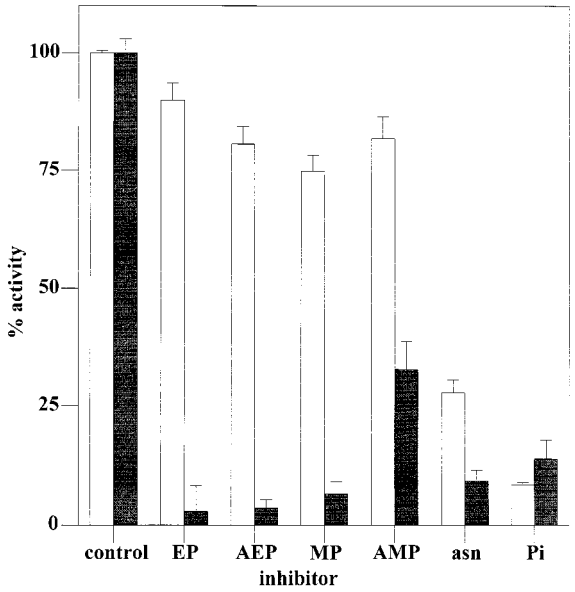

FIG. 6. The Pit system is the major P_i uptake system under conditions of excess P_i. The bar graph depicts the inhibition of phosphate uptake in strain Rm1021 (wild type) ▭ and strain RmG804 (*pit310*::Tn*5*) ▬ by various inhibitors. Inhibition experiments were carried out at a phosphate concentration of 4 μM and an inhibitor concentration of 50 μM on cells grown in medium supplemented with 2 mM P_i. 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 Rm1021 and 0.2 nmol/min/mg of protein for strain RmG804. Abbreviations: control, no addition; EP, ethylphosphonate; AEP, aminoethylphosphonate; MP, methylphosphonate; AMP, aminomethylphosphonate; asn, arsenate. AP and succinate uptake values for this experiment were 9.0 U and 40.1 ± 1.4 nmol/min/mg of protein for strain Rm1021 and 8.9 U and 36.9 ± 1.2 nmol/min/mg of protein for strain RmG804, respectively.

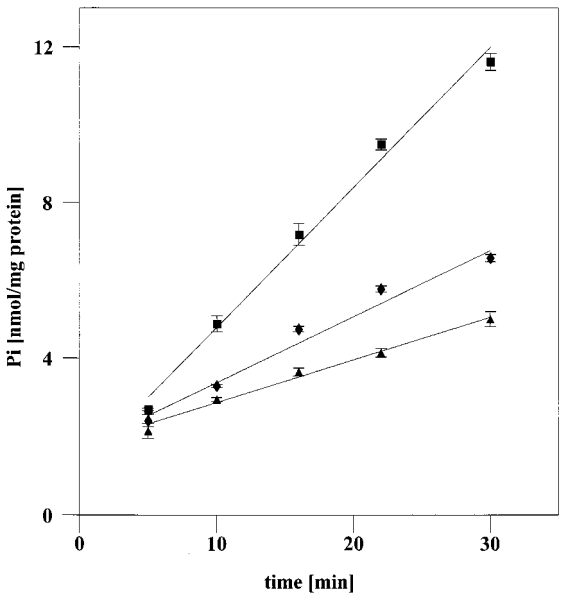

FIG. 7. The activity of the Pit system is reduced in a *phoCDET* mutant strain. The time course of phosphate uptake in the parental strain Rm1021 (■), the *phoC* mutant RmG490 (◆), and the *phoC pit* double mutant RmG830 (▲) is shown. Uptake activity was assayed at a substrate concentration of 4 μM with cells grown in medium supplemented with 2 mM phosphate. The succinate uptake values for this experiment were 44.8 ± 0.3 nmol/min/mg of protein for strain Rm1021, 62.2 ± 1.0 nmol/min/mg of protein for strain RmG490, and 52.6 ± 2.5 nmol/min/mg of protein for strain RmG830.

(Fig. 6). While the absolute transport rate was much lower than for wild-type cells or *pit* mutants grown under phosphate starvation (Fig. 1 and data not shown), the response to the addition of phosphonates, arsenate, and excess unlabeled P_i was virtually identical to that observed for the PhoCDET system in phosphate-starved wild-type cells (Fig. 2 and 6). These data showed that there was no additional P_i uptake system with a substrate specificity similar to the Pit system functioning in *R. meliloti* cells. We therefore conclude that the Pit uptake system is used for P_i uptake in *R. meliloti* wild-type cells growing in medium containing high concentrations of phosphate.

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* raised the question why *phoCDET* mutants grow slowly in medium containing 2 mM P_i as the sole phosphorus source (Fig. 5B) (4). Since the Pit uptake system appears to be used exclusively for P_i uptake into wild-type *R. meliloti* cells growing in medium containing 2 mM P_i, 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 (*phoCΩ490*), and the double mutant RmG830 (*phoCΩ490 pit310*::Tn*5*) following growth of the cells in medium containing 2 mM phosphate (Fig. 7). With a substrate concentration of 4 μM, we found that the rate of P_i 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 can be explained by the presence of an uncharacterized uptake system(s) which is capable of transporting P_i 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 P_i transport. The Pit protein is encoded by the *orfA-pit* operon, and the possible involvement of the OrfA protein in P_i transport has not yet been investigated (3).

The kinetic parameters determined for the PhoCDET system were a K_m of 0.2 μM and a V_{max} of 6.8 nmol/min/mg of protein. The low K_m 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 K_m of 0.4 μM and a V_{max} 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 (*phoCΩ490*) in medium containing ethylphosphonate as the sole source of phosphorus (Fig. 5B) (4). However, the ability of the *phoC* mutant RmG490 to grow on other phosphonates demonstrates that phosphonate uptake systems in addition to PhoCDET are present in *R. meliloti* (see below).

The K_m value of about 1.8 μM for P_i 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 P_i transport to inhibition by phosphonates, indicates that Pit is a low-affinity, high-specificity P_i uptake system (Fig. 3 and 4). 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 (K_m, 38.2 μM; V_{max}, 55 nmol/min/mg [dry weight]) (25). In *E. coli*, P_i 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 Mg^{2+} and Ca^{2+} 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 Ca^{2+} or Mg^{2+} in the assay mixture (data not shown).

The biochemical properties of the *R. meliloti* P_i uptake systems 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 P_i (2 mM), *orfA-pit* is expressed and the OrfA-Pit transport system is

used for P_i transport, whereas the *phoCDET* genes are not expressed. We showed that in medium containing 2 mM P_i, *phoCDET* mutants behave as though they are phosphate starved and *orfA-pit* expression is very low. Both the *sfx1* and *phoB* mutations were shown to increase *orfA-pit* transcription (2, 3). Our present data showing an identical substrate specificity of P_i uptake in strain RmG762 (*phoCΩ490 sfx1*) and strain RmH838 (*phoB3*::Tn*V*) (Fig. 4) suggest that the same transporter is being used in both of these strains. Further, the specificity of the P_i uptake system used in these strains is identical to the substrate specificity observed in wild-type cells grown in medium containing 2 mM P_i. Thus, the biochemical data are entirely consistent with the genetic data suggesting that the *orfA-pit* operon encodes a P_i transport system which is expressed in *R. meliloti* cells growing in the presence of excess P_i.

We have previously reported that whereas *phoC* mutants grow poorly in medium containing 2 mM P_i (4), *phoC pit* double mutants show a further decrease in their growth rate (3). In this study, we measured P_i uptake in the wild type, Rm1021, the *phoC* mutant RmG490 (*phoCΩ490*), and the double mutant RmG830 (*phoCΩ490 pit310*::Tn*5*) (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 growth rates in medium containing 2 mM P_i is simply a result of a reduction in P_i uptake as opposed to some other aspect of P_i assimilation. Since *phoC* mutants grown in 2 mM P_i are phenotypically phosphate starved, it is very likely that the signal transduction pathway regulating the expression of both P_i 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 P_i). We have not been able to determine the level of Pit activity in phosphate-starved wild-type cells; however, we believe that the activity of the Pit system determined in the *phoC* mutant grown in the presence of excess P_i (Fig. 7) does reflect the activity state of Pit 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 (*phoB3*::Tn*V*) to grow on phosphonates (except aminoethylphosphonate) might suggest that the alternate phosphonate 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 aminoethylphosphonate 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 aminoethylphosphonate 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 P_i-limited chemostat cultures of *R. meliloti* WU3 and *Rhizobium* sp. strain NGR234 took up P_i at least 10 times faster than phosphate-rich cultures did. The K_m for P_i uptake in the P_i-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 P_i uptake in P_i-limited cells of *R. meliloti* Rm1021 is much higher than the uptake rate in P_i-sufficient cells, the K_m determined for the P_i-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 P_i uptake activities (0.6 to 1.4 nmol/min/mg of protein) measured for cells grown in medium containing 2 mM P_i 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 P_i uptake in P_i-sufficient cells and bacteroids of *Rhizobium tropici*. Moreover, in many of the experiments, we observed that despite the apparent low rates of P_i 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 P_i uptake systems is common 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 P_i transport in *E. coli* remains to be established.

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