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Characterization of Two Inducible Phosphate Transport Systems in *Rhizobium tropici*

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Rhizobium tropici **forms nitrogen-fixing nodules on the roots of the common bean (*Phaseolus vulgaris*). Like other legume-*Rhizobium* symbioses, the bean-*R. tropici* association is sensitive to the availability of phosphate (P_i). To better understand phosphorus movement between the bacteroid and the host plant, P_i transport was characterized in *R. tropici*. We observed two P_i transport systems, a high-affinity system and a low-affinity system. To facilitate the study of these transport systems, a Tn*5*B22 transposon mutant lacking expression of the high-affinity transport system was isolated and used to characterize the low-affinity transport system in the absence of the high-affinity system. The K_m and V_{max} values for the low-affinity system were estimated to be 34 ± 3 μM P_i and 118 ± 8 nmol of $P_i \cdot min^{-1} \cdot mg$ (dry weight) of $cells^{-1}$, respectively, and the K_m and V_{max} values for the high-affinity system were 0.45 ± 0.01 μM P_i and 86 ± 5 nmol of $P_i \cdot min^{-1} \cdot mg$ (dry weight) of $cells^{-1}$, respectively. Both systems were inducible by P_i starvation and were also shock sensitive, which indicated that there was a periplasmic binding-protein component. Neither transport system appeared to be sensitive to the proton motive force dissipator carbonyl cyanide *m*-chlorophenylhydrazone, but P_i transport through both systems was eliminated by the ATPase inhibitor *N,N′*-dicyclohexylcarbodiimide; the P_i transport rate was correlated with the intracellular ATP concentration. Also, P_i movement through both systems appeared to be unidirectional, as no efflux or exchange was observed with either the wild-type strain or the mutant. These properties suggest that both P_i transport systems are ABC type systems. Analysis of the transposon insertion site revealed that the interrupted gene exhibited a high level of homology with *kdpE*, which in several bacteria encodes a cytoplasmic response regulator that governs responses to low potassium contents and/or changes in medium osmolarity.**

Nitrogen fixation in legume nodules involves a complex exchange of nutrients between the plant and bacteroids. This exchange involves transport across the bacteroid membrane and the plant-derived envelope surrounding the bacteroid, the peribacteroid membrane. In its simplest terms, this symbiosis is often viewed as an exchange of reduced carbon for reduced nitrogen. However, it is clear that optimum nodule function also involves a balanced flow of other nutrients (33). One nutrient that has been shown to be important for this symbiosis is phosphorus. Low phosphorus availability in soils is common and limits legume production worldwide; however, phosphorus metabolism in this plant-microbe interaction has not been well characterized. Given the significant metabolic activity of bacteroids, the phosphorus supply may be critical for optimum symbiotic functioning of bacteroids, and understanding the mechanisms by which bacteroids acquire phosphorus should provide useful information concerning phosphorus exchange between the symbionts and phosphorus flow in the symbiosis.

Phosphate (P_i) uptake has been investigated in various bacteria. In some microorganisms only a single transport system has been found. This is the case for *Micrococcus lysodeikticus* (19) and for several *Rhizobium* species (41). In other bacteria, two P_i transport systems have been found. Examples of such bacteria include *Escherichia coli* (34, 53), *Acinetobacter johnsonii* (48), and *Pseudomonas aeruginosa* (26). In each of the latter bacteria, a constitutively expressed low-affinity transport system and a P_i-repressible high-affinity permease have been identified. In *E. coli*, the low-affinity P_i transport system (LATS) is energized by the proton motive force (Δp) and consists of a single membrane component (17). In contrast, the high-affinity P_i transport system (HATS) is a multicomponent system consisting of proteins associated with the cytoplasmic membrane, an ATP-binding protein, and a periplasmic solute-binding protein (reviewed in reference 51).

Recently, *Sinorhizobium meliloti* has been reported to have at least two P_i transport systems, consistent with the high-affinity–low-affinity model described above (49). The high-affinity system is encoded by the *phoCDET* operon, and the low-affinity system is encoded by *pit* (in the *orfA-pit* operon) (6). Previously published evidence strongly suggests that expression of the genes coding for both P_i transport systems in *S. meliloti* is controlled by PhoB (6). PhoB (presumably phosphorylated PhoB) positively regulates the *phoCDET* operon but negatively controls *orfA-pit*. Under nonlimiting P_i conditions, the low-affinity Pit permease is expressed and is primarily responsible for P_i uptake. When *S. meliloti* is grown under P_i-limiting conditions, the Pit system is repressed, while the high-affinity PhoCDET system is induced and becomes the primary mechanism of P_i transport.

Some of our efforts to characterize and understand phosphorus metabolism and exchange in the *Rhizobium*-legume association have focused on the *Rhizobium tropici*-bean symbiosis (1), with initial work aimed at characterizing P_i assimilation and regulation in the microbial partner. As observed with other gram-negative bacteria (51), *R. tropici* induces alkaline phosphatase, and its P_i transport rate increases significantly in response to P_i limit-limiting conditions (1). The induction occurs when the medium P_i concentration is approximately 1 μM (1). *R. tropici* bacteroids isolated from

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nodules of bean plants grown in the presence of nonlimiting phosphorus concentrations contain extremely high levels of alkaline phosphatase, as well as a P_i stress-inducible acid phosphatase (1). This implies that under normal growth conditions a bean plant provides very low levels of P_i to the bacteroids in its nodules. In order to determine the importance of P_i supply for the bean-bacteroid symbiotic system, we are now assessing *R. tropici* P_i transport systems and estimating their kinetic properties. In this report, the P_i transport systems of *R. tropici* are described. Like *S. meliloti* (49), this bacterium has two distinct functional P_i transport systems. However, *R. tropici* appears to differ from *S. meliloti* and all other bacteria investigated previously since both P_i transport systems are inducible by P_i stress, are shock sensitive, and are energized by phosphate bond energy. In addition, in this paper we also describe a mutant that lacks high-affinity P_i transport activity.

MATERIALS AND METHODS

Strains and culture conditions. Strains CIAT899 and CAP45 were used in all experiments. CIAT899 is the type strain of *R. tropici* type IIB (29), and CAP45 is a P_i transport mutant derived from CIAT899 (see below). CIAT899 was maintained on the minimal mannitol-ammonium agar ($MMNH_4$) (pH 7.2) described previously (42, 43). CAP45 was maintained on the same medium, except that β-glycerolphosphate (βGP) replaced mannitol as the sole carbon source and gentamicin was included at a final concentration of 25 mg · liter^{-1}. The other antibiotics used in the experiments were ampicillin (100 mg · liter^{-1}) and tetracycline (25 mg · liter^{-1}). In experiments in which P_i-starved cells ($-P_i$ cells) were used, the cells were incubated in $MMNH_4$ which lacked added phosphorus but was buffered to pH 7.2 with 5 mM MES (morpholineethanesulfonic acid) and 10 mM MOPS (morpholinepropanesulfonic acid) ($MMNH_4$-OP) (43).

Mutant isolation. Pho regulatory mutants that are constitutive for the P_i-repressible alkaline phosphatase often also do not express a high-affinity P_i transporter (5; reviewed in reference 51). We used the strategy and methods of Torriani and Rothman (44) to isolate *R. tropici* mutants that expressed alkaline phosphatase constitutively and then screened these mutants for a P_i transport phenotype. Briefly, *R. tropici* CIAT899 was mutated with transposon Tn*5*B22 (40) as previously described (2, 15). *E. coli* S17-1 (39) was used to mobilize the transposon into CIAT899 in mating mixtures, which were plated onto βGP-gentamicin agar. In order to be used as a carbon source, βGP must first be dephosphorylated at rates sufficient to supply glycerol for growth. One candidate phosphatase in CIAT899 is alkaline phosphatase (1). However, because of the high concentration of P_i in the medium, growth would require *phoA* expression under conditions where this gene is normally repressed. Transconjugants were isolated from the βGP-gentamicin agar plates by streaking twice to obtain pure cultures, and then constitutive expression of alkaline phosphatase activity was measured by comparing alkaline phosphatase activities in cells grown under high-P_i conditions ($MMNH_4$ broth) and in cells after incubation under zero-P_i conditions ($MMNH_4$-OP broth). Periplasm proteins were extracted and alkaline phosphatase was assayed by using previously described methods (1). Subsamples of the mutants were then screened for P_i uptake to identify mutants that were defective in P_i transport.

Transport assays. Early-stationary-phase $MMNH_4$ cultures were washed twice in $MMNH_4$-OP and resuspended in $MMNH_4$-OP to an optical density of 0.60 at an absorbance of 595 nm. To obtain $-P_i$ cells, washed cells were incubated in $MMNH_4$-OP at 30°C for 7 h in order to allow for maximum induction of P_i transport (1). Chloramphenicol (50 mg · liter^{-1}) was then added to stop further protein synthesis. Cells not starved for P_i ($+P_i$ cells) were prepared in the same way except that chloramphenicol was added immediately after washing and the cells were used within 1 h. In preliminary experiments, we found that chloramphenicol did not interfere with P_i transport (results not shown) but did inhibit the synthesis of alkaline phosphatase for at least 5 h. Thus, we concluded that de novo protein synthesis in $+P_i$ cells did not occur during the experiments performed with $+P_i$ cells.

The standard transport assay was conducted in an orbital shaker water bath at 30°C. Washed cells were diluted with $MMNH_4$-OP to a concentration of 0.025 mg (dry weight) of cells · ml^{-1} for $-P_i$ cells. Because the P_i transport rates were much lower in $+P_i$ cells, the cell concentration used in $+P_i$ cell assays was 0.125 mg (dry weight) of cells · ml^{-1} to ensure that sensitive and accurate uptake measurements were obtained. After 5 min of preincubation in $MMNH_4$-OP, the transport assay was initiated by adding P_i (at concentrations specified below) as [^{32}P]KH_2PO_4 (specific activity, 22.5 μCi · μmol^{-1}). The [^{32}P]KH_2PO_4-containing solution was filtered prior to use in order to remove any extraneous particles that had adsorbed label. Cell samples (0.5 ml) were withdrawn at 20-s intervals (unless otherwise specified); each sample was collected on a 0.3-μm-pore-size glass fiber filter (Gelman Sciences, Ann Arbor, Mich.) and washed with 20 ml of transport rinse buffer, which contained 20 mM MES and 5 mM KH_2PO_4 (pH 6.5). The filters were placed in counting vials, 20 ml of H_2O was added to each vial, and the radioactivity retained on the filters was measured as Cerenkov radiation (21). All counts were corrected for background values and were standardized by using similarly prepared spiked standard samples.

Phosphate exchange and efflux. The methods of Medveczky and Rosenberg (30) were modified slightly for use with *R. tropici*. Briefly, $-P_i$ cells were loaded for 4 min with [^{32}P]KH_2PO_4 (either 5 or 400 μM; specific activity, 22.5 μCi · μmol^{-1}) at 30°C and then diluted 100-fold with $MMNH_4$-OP without unlabeled potassium phosphate (efflux experiments) or with either 25 μM or 2 mM unlabeled potassium phosphate (exchange experiments). At time intervals, 0.5-ml samples were filtered and washed with transport rinse buffer as described above for the transport experiments.

Osmotic shock treatment. An osmotic shock procedure similar to that described by Neu and Heppel (31) was used. Cells were washed twice with 30 mM Tris (pH 8.0) and resuspended to a density of 5 mg (dry weight) of cells · ml^{-1} in 30 mM Tris (pH 8.0) containing 1 M sucrose and 10 mM EDTA. Following 15 min of incubation at room temperature, cells were collected by centrifugation for 4 min at 14,000 × *g*, and then periplasmic proteins were released by resuspending the pellet in 0.1 mM $MgSO_4$ at room temperature. The shock-treated cells were collected by centrifugation, gently resuspended in $MMNH_4$-OP, and then used for P_i transport assays.

To verify that periplasmic enzymes were released, the protein concentrations and levels of activity of the periplasm marker enzyme alkaline phosphatase in the supernatant of the pelleted shock-treated cells were determined. In addition, the cytoplasm marker enzyme malate dehydrogenase was assayed to determine if cell lysis had occurred. We also determined the alkaline phosphatase and protein levels in supernatants of pelleted control cells and in cleared extracts of sonicated samples that contained equivalent amounts of shocked cells. Alkaline phosphatase activity was measured as described above, and malate dehydrogenase activity was assayed at 340 nm by determining the rate of NADH oxidation (1). Each 350-μl reaction mixture contained 1.5 mM oxalacetic acid, 0.25 mM NADH, 10 mM K_2HPO_4 (pH 7.5), and 50 μl of shock fluid or cell extract (1). Both assays were conducted with a Bio-Rad model 3550-UV microplate reader. Protein concentrations were determined by using a Bio-Rad protein assay kit.

EDTA treatment of cells. Like previous investigators (23, 25, 48), we found that it was necessary to use a mild EDTA treatment to permeabilize the outer membrane in order to use the ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD), the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the Δp probe tetraphenylphosphonium bromide (TPP^+). $-P_i$ cells were washed twice with 30 mM Tris (pH 8.0) and resuspended to a density of 5 mg (dry weight) of cells · ml^{-1} in 30 mM Tris, and then 1 mM EDTA (pH 7.0) was added. After 5 min of incubation at room temperature, the cells were centrifuged for 4 min at 14,000 × *g*, washed twice with 30 mM Tris, and resuspended in $MMNH_4$-OP to a density of 0.5 mg (dry weight) of cells · ml^{-1}. Alkaline phosphatase was not released by this procedure (data not shown), which indicated that the EDTA treatment did not result in release of periplasmic proteins.

Energy coupling. (i) Qualitative determination of membrane potential. CCCP was used to dissipate all components of the Δp (23, 24). Δp probes, such as TPP^+, are passively distributed between the cell and the medium depending on the membrane potential (25) and can be used to assess the effect of CCCP on Δp (23, 25, 35). TPP^+ uptake was measured with and without CCCP by using the medium and conditions described above for P_i uptake, except that choline chloride was added to a final concentration of 50 mM. Choline chloride reduces binding of TPP^+ to anionic groups at the cell surface (28) but does not interfere with P_i uptake (data not shown).

For the TPP^+ uptake assays we used $MMNH_4$-OP with or without CCCP (final concentration, 1 μmol of CCCP per 0.025 mg [dry weight] of EDTA-treated $-P_i$ cells per ml). Two types of CCCP addition experiments were performed. In the first type, CCCP was added to a cell suspension 5 min before [^{3}H]TPP^+ (final concentration, 18 μM; specific activity, 27.5 μCi · μmol^{-1}) was added. After [^{3}H]TPP^+ was added, 0.5-ml cell samples were removed at specific times and then collected and washed with 0.3-μm-pore-size glass fiber filters as described above for the P_i transport experiments. In the second type of experiment, [^{3}H]TPP^+ was added to initiate the transport assay, the cells were allowed to accumulate [^{3}H]TPP^+ for 4 min, and then CCCP was added after 4.5 min; this was followed by cell sampling. For both types of experiments, the [^{3}H]TPP^+ content of the cells was measured by placing the filters in counting vials, adding 20 ml of scintillation cocktail (Scintisafe Plus 50%; Fisher Chemical) to each vial, and measuring the radioactivity with a Tri-Carb liquid scintillation analyzer (model 4430; Packard Instrument Co.). All counts were corrected for background values and were standardized by using similarly prepared spiked standard samples.

In experiments performed to determine the effect of CCCP on P_i transport, P_i transport assays were performed as described above for the routine assays, except that the cells were incubated in the presence of CCCP (1 μmol of CCCP per 0.025 mg [dry weight] of EDTA-treated $-P_i$ cells per ml) for 5 min at 30°C with constant shaking before [^{32}P]KH_2PO_4 (specific activity, 22.5 μCi · μmol^{-1}; same concentration as described above) was added. At specific times, cell samples were removed and filtered, and radioactivity was quantified as described above. [^{3}H]TPP^+ was not included in the assay mixtures.

To separately manipulate ATP pools and Δp for P_i transport assays, mixtures containing CCCP were preincubated for 5 min, which resulted in dissipation of the membrane potential without appreciable reductions in the ATP pool size

TABLE 1. Effect of Δp dissipation and ATP depletion on P_i transport in *R. tropici* CIAT899 and CAP45[a]

Treatment	Strain	P_i uptake (nmol of P_i/min/mg [dry wt] of cells)		ATP concn (nmol of ATP/mg [dry wt] of cells)
		5 μM P_i	400 μM P_i	
Ethanol control	CIAT899	26.7 ± 3.2	41.8 ± 3.1	2.20 ± 0.20
	CAP45	2.4 ± 0.3	8.8 ± 0.1	1.41 ± 0.31
CCCP (1 μM)	CIAT899	23.8 ± 1.4 (11)[b]	44.9 ± 2.1 (0)	1.84 ± 0.08 (18)
	CAP45	2.1 ± 0.1 (12)	12.2 ± 0.1 (0)	1.35 ± 0.26 (4)
Ethanol control	CIAT899	10.2 ± 2.2	11.76 ± 1.97	0.305 ± 0.005
	CAP45	2.2 ± 0.1	7.06 ± 0.37	1.03 ± 0.16
DCCD (100 μM)	CIAT899	0 ± 0 (100)	0.43 ± 0.08 (96)	0.02 ± 0.01 (93)
	CAP45	0.034 ± 0.001 (98)	0.29 ± 0.02 (96)	0.09 ± 0.04 (91)

[a] $-P_i$ cells were treated with EDTA as described in the text, preincubated with an inhibitor and then assayed to determine P_i uptake and the intracellular ATP concentration. Values are means ± standard errors based on values from two separate experiments. Cells were preincubated for 5 min in the presence of the Δp dissipator CCCP or for 45 min in the presence of the ATPase inhibitor DCCD. Using ethanol to solubilize CCCP and DCCD did have a negative effect on the overall transport rates, and therefore an ethanol control was included in all experiments.

[b] The values in parentheses are percentages of reduction in P_i transport or ATP concentration compared to the controls.

(i.e., we avoided reductions in the ATP pool size via loss of H^+-ATPase function). To facilitate exhaustion of intracellular ATP pools, cells were preincubated in the presence of DCCD for 45 min (see below). Short incubations (5 min) in transport suspension media that contained ethanol (final concentration, 0.8% [vol/vol]; ethanol was required to solubilize CCCP and DCCD) were found to have negative effects on the rates of uptake by both the HATS and LATS in *R. tropici* (compare the rates in Table 1 to the estimated V_{max} values in Table 2). Prolonged incubation (45 min) further reduced the rates of uptake by the HATS but appeared to have no additional effect on the LATS (Table 1).

(ii) Determination of ATP concentrations. Intracellular ATP concentrations were determined in experiments in which the effects of CCCP and DCCD were examined. To determine ATP concentrations, the reaction mixtures used were identical to the P_i transport assay reaction mixtures, except that no radioisotope was added. After incubation (see below), cellular ATP was extracted as described by Joshi et al. (23). ATP concentrations were determined by using the luciferase assay, measuring light emission with a Turner model TD-20e luminometer, and employing the internal standard technique (47). Each ATP assay mixture contained 0.05 ml of perchloric acid-treated supernatant, 0.1 ml of 10 mM Tris buffer (pH 8.0), and 0.1 ml of luciferase-luciferin (Promega). After luciferase was injected into the sample and light was measured, an ATP standard was added to the same cuvette and the light was measured again. The amount of ATP in the sample was calculated by using the following equation: ATP concentration = [(RU − RB)/(RIS − RU)] × ATP concentration in the standard, where RU is the luminescence value for the sample, RB is the luminescence value for the blank, and RIS is the luminescence value after addition of the internal standard.

Nucleic acid manipulations. The protocols of Sambrook et al. (36) were used for all routine manipulations of plasmid and chromosomal DNA. The Tn*5*B22 insertion site in the mutant was characterized by selectively subcloning the transposase portion of Tn*5*B22 (40) containing the gentamicin resistance gene along with the balance of the transposon and flanking chromosomal DNA. The transposon-chromosome junction was then sequenced, and the resulting nucleotide sequence data were used to conduct a BLASTX search to identify a possible match (4, 16). Briefly, total chromosomal DNA was harvested from the mutant, digested with *Xma*I, and then ligated into pBluescript KS(+) (Stratagene). The ligation mixture was transformed into *E. coli* DH5α (36), and plasmids from transformants that were resistant to ampicillin and gentamicin were analyzed by restriction analysis to verify that each contained a single cloned fragment. Southern blotting was then used to verify that the cloned fragment was identical to the fragment in the genome of mutant CAP45. The flanking DNA was sequenced by using an ABI Prism BigDye kit (PE Applied Biosystems, Foster City, Calif.) and an ABI model 310 genetic analyzer (PE Applied Biosystems). The primer 5′-CCATGTTAGGAGGTCACATGGAAGT-CAG-3′ was used to initiate sequencing from the transposase terminal (40).

RESULTS

Isolation of phosphate transport mutant CAP45. Tn*5*B22 mutagenesis of *R. tropici* CIAT899 and selection on minimal βGP-gentamicin agar resulted in several gentamicin-resistant mutants that were found to be constitutive for expression of alkaline phosphatase. Enzyme assays of periplasmic extracts obtained from these mutants revealed that they had alkaline phosphatase specific activities of about 800 U (1 U = 1 nmol of *p*-nitrophenylphosphate hydrolyzed · min^{-1} · mg of $protein^{-1}$) when $+P_i$ cells were used. Typically, the alkaline phosphatase activity of CIAT899 $+P_i$ cells is approximately 30 U, and the alkaline phosphatase activity of CIAT899 $-P_i$ cells is approximately 1,500 U (1). Presumably, constitutive expression of alkaline phosphatase in the mutants was due to a lack of normal repressive regulatory mechanisms. By screening a subset of the mutants for a P_i transport phenotype we identified isolates that had reduced P_i transport rates. Southern blot analysis of chromosomal DNA prepared from the P_i transport mutants verified that Tn*5*B22 was present, and all of the blot patterns appeared to be identical, suggesting that the insertion sites were very similar or that the mutants were siblings (results not shown). One representative isolate of these transport mutants was selected for further study; this isolate was designated CAP45.

Kinetic parameters of phosphate uptake. Kinetic plots of P_i transport in both $+P_i$ cells and $-P_i$ cells of CIAT899 revealed that two separate transport systems were present. Eadie-Hofstee plots of P_i transport in $-P_i$ cells of CIAT899 and CAP45 are shown in Fig. 1. As measured at P_i concentrations of 0.1 to 500 μM and calculated from a linear regression analysis, the estimated K_m values for two transport systems differed by approximately 2 orders of magnitude. In addition to being expressed under high-P_i growth conditions, both systems were induced in response to P_i deprivation, as shown by the increases in the V_{max} values of $-P_i$ cells (Table 2).

Only a single transport system was evident in CAP45 (Fig. 1 and Table 2), providing an opportunity to study it in the absence of the other system that would otherwise influence over-

TABLE 2. Kinetic parameters of P_i uptake in *R. tropici* CIAT899 and CAP45[a]

Strain	Growth conditions	High-affinity uptake		Low-affinity uptake	
		K_m (μM P_i)	V_{max} (nmol of P_i/min/mg [dry wt] of cells)	K_m (μM P_i)	V_{max} (nmol of P_i/min/mg [dry wt] of cells)
CIAT899	$-P_i$	0.45 ± 0.01	86.2 ± 4.9	9.6 ± 1.0	153.8 ± 13.6
	$+P_i$	0.34 ± 0.02	0.22 ± 0.01	35.7 ± 1.0	1.33 ± 0.01
CAP45	$-P_i$	ND[b]	ND	34.3 ± 2.7	118.0 ± 7.5
	$+P_i$	ND	ND	33.1 ± 1.3	0.41 ± 0.02

[a] The kinetics of P_i uptake were analyzed by using Eadie-Hofstee plots. The initial velocities in $+P_i$ and $-P_i$ cells were determined for the first 20 and 10 s, respectively. The values are means ± standard errors based on values from three separate experiments; each P_i concentration was replicated three times in each experiment.

[b] ND, not detected.

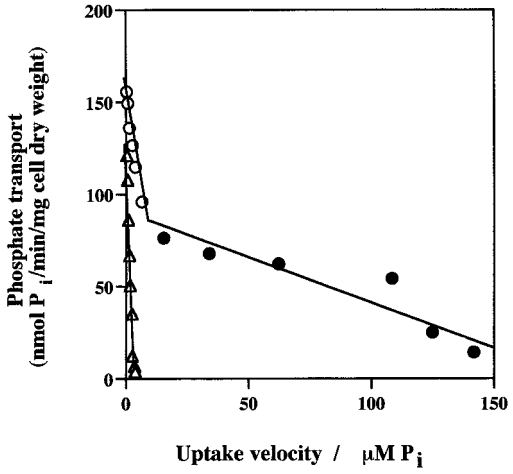

FIG. 1. Eadie-Hofstee plots of initial P_i uptake velocities in *R. tropici* CIAT899 and CAP45. Symbols: ●, CIAT899 HATS; ○, CIAT899 LATS; △, CAP45 LATS. Transport rates were determined with $-P_i$ cells as described in the text at P_i concentrations between 0.1 and 500 μM. Each point is the mean of values from three independent experiments; for each experiment three replicate values were obtained at each P_i concentration. The standard error for each data point did not exceed 10% of the mean, and the standard errors are not shown to simplify presentation.

all P_i transport behavior. The K_m for this P_i permease was found to be 34 μM, which suggested that the system was the low-affinity system present in CIAT899. The V_{max} for the low-affinity system present in CAP45 was similar to the V_{max} obtained for CIAT899 under both $+P_i$ and $-P_i$ growth conditions (Fig. 1 and Table 2). However, the proportional increase in the estimated V_{max} for CAP45 $-P_i$ cells suggested that the increase in P_i transport by the low-affinity system in response to P_i stress was more substantial than the increase observed in wild-type strain CIAT899. Based on the estimated K_m values, P_i concentrations of 5 and 400 μM were used in subsequent experiments to evaluate P_i uptake by the HATS and the LATS, respectively, when the effects of various treatments or inhibitors were determined.

Effect of osmotic shock on phosphate uptake. Osmotic shock release of periplasmic proteins was used to determine if either P_i transport system required a periplasmic solute-binding protein to exhibit the maximal transport rate. P_i uptake was dramatically reduced in osmotic shock-treated cells (Fig. 2). As determined with high and low P_i levels that were saturating for either P_i transport system, osmotic shock reduced the P_i transport rates by approximately 80%. This was the case for both strains and suggested that both the HATS and the LATS depend on a P_i-binding protein for maximal P_i translocating activity.

Periplasmic protein release and the structural integrity of osmotically shocked cells were verified by assaying for the marker enzymes alkaline phosphatase and malate dehydrogenase, respectively. The supernatant of pelleted shocked $-P_i$ cells contained 19% of the total cellular protein and 16% of the total alkaline phosphatase activity (Table 3). The protein concentration and alkaline phosphatase activity in the supernatant obtained from the same quantity of pelleted nonshocked control cells were less than 1% of the values in the supernatant of pelleted shocked cells. The combination of relatively high levels of alkaline phosphatase and the presence of proteins in the supernatant of the shock-treated cells was taken as evidence that the cells lost significant amounts of periplasmic proteins during the osmotic shock treatment. The complete lack of detectable malate dehydrogenase activity in the shock fluids also demonstrated that the shock treatment did not lyse the cells (Table 3).

Energy coupling to phosphate transport. To assess the roles of Δp and ATP in energizing P_i transport, CIAT899 and CAP45 were treated with CCCP and DCCD. The protonophore CCCP dissipates the energized membrane and inhibits processes that use the Δp directly as a source of energy (i.e., secondary transport systems). However, reactions driven directly by phosphate bond energy should be relatively resistant to the action of this compound. Conversely, the ATPase inhibitor DCCD should significantly reduce ATP levels, and thus ATP-dependent transport activity should also be significantly reduced when DCCD is added. On the basis of these criteria, we examined energy coupling to P_i transport in both CIAT899 and CAP45. Under the conditions used in the assays (pH 7.2), neutrophilic bacteria, such as rhizobia, do not generate a significant chemical potential (ΔpH), and therefore the Δp consists primarily of the electrical membrane component ($\Delta\Psi$) (25), which in cowpea rhizobia has been shown to be unaffected by changes in pH (20).

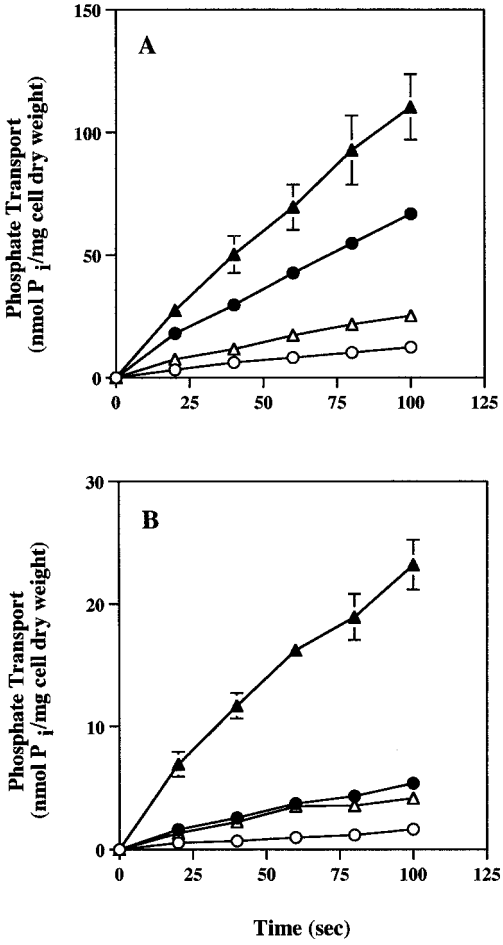

FIG. 2. Effect of osmotic shock on the uptake of P_i in $-P_i$ cells of *R. tropici* CIAT899 and CAP45. P_i uptake was determined for both control cells and shocked cells at P_i concentrations of 5 μM and 400 μM. (A) P_i uptake in wild-type strain CIAT899. (B) P_i uptake in mutant CAP45. Symbols: ○, 5 μM P_i with shocked cells; ●, 5 μM P_i with control cells; △, 400 μM P_i with shocked cells; ▲, 400 μM P_i with control cells. The value for each time point is the mean of values from three independent experiments for CIAT899 or two independent experiments for CAP45. The error bars indicate the standard errors of the means.

TABLE 3. Release of periplasmic proteins from CIAT899 by osmotic shock treatment[a]

Treatment	Protein concn (μg of protein/ml of extract)	Alkaline phosphatase activity (nmol/min/ml of extract)[b]	Malate dehydrogenase activity (nmol/min/ml of extract)[c]
Control cells	0.5 (0.1)[d]	15 (0.8)	0 (0)
Osmotically shocked cells	54.5 (19.3)	182 (15.6)	0 (0)
Sonicated cells (control)	354.3 (99.9)	1,880 (99.2)	321 (100)
Sonicated cells (shocked)	228.6 (80.7)	983 (84.4)	284 (100)

[a] $-P_i$ cells were shocked as described in the text. Control cells were treated like the shocked cells were treated, except that neither EDTA nor sucrose was used. The data are data from a single representative experiment.

[b] Nanomoles of *p*-nitrophenyl phosphate hydrolyzed per minute per milliliter of extract.

[c] Nanomoles of NADH oxidized per minute per milliliter of extract.

[d] The values in parentheses are percentages of the total cellular protein or enzyme activity.

The effects of CCCP on Δp, as measured by uptake and accumulation of the Δp probe [^{3}H]TPP$^+$, are shown in Fig. 3. In one set of experiments, CCCP was included in each cell suspension before [^{3}H]TPP$^+$ was added during the uptake assay (Fig. 3A). These experiments showed that CCCP dissipated Δp, which resulted in significantly reduced [^{3}H]TPP$^+$ uptake and accumulation. In other experiments, CCCP was added to cells that were in the process of accumulating [^{3}H]TPP$^+$. This addition resulted in the immediate release of [^{3}H]TPP$^+$; again, the data showed that CCCP treatment dissipated a significant portion of the Δp (interior negative) but also demonstrated that [^{3}H]TPP$^+$ did not simply bind to cell components, as it was readily released when the Δp was dissipated. On the basis of several such experiments in which CCCP treatment consistently either resulted in the release of [^{3}H]TPP$^+$ or inhibited [^{3}H]TPP$^+$ uptake and accumulation by 50 to 90% compared to control cells, we concluded that CCCP largely eliminated the Δp and could be used to assess the importance of the Δp as the driving force for P_i transport in *R. tropici*.

As shown in Table 1, under P_i transport assay conditions identical to the conditions used in the experiments whose results are shown in Fig. 3A (which verified that CCCP significantly reduced the Δp), CCCP treatment of cells had no effect on P_i transport with either transport system compared to cells not treated with CCCP. As expected, the ATP levels in cells treated with CCCP under these conditions were also not affected. In contrast to CCCP treatment, DCCD treatment reduced the ATP levels to near zero and eliminated P_i transport in both CIAT899 (which contains both transport systems) and CAP45 (which contains only the LATS) (Table 1). P_i transport rates were highly positively correlated with ATP levels in the cell ($r^2 = 0.95$ for the HATS in CIAT899; $r^2 = 0.89$ for the LATS in CAP45). These results indicate that the Δp per se is not involved in energizing P_i transport by either system. Rather, the correlation between ATP levels and P_i transport suggests that ATP is involved in energizing both P_i transport systems.

Exchange and efflux of phosphate. After dilution of preloaded cells with media containing no P_i or with media containing excess unlabeled P_i, the level of radioactivity in CIAT899 remained constant, implying that neither P_i transport system mediated P_i efflux or exchange of internal P_i with external P_i (Fig. 4A and B). Mutant strain CAP45 behaved similarly (Fig. 4C). CIAT899 cells preloaded with 400 μM [^{32}P]KH_2PO_4 (to evaluate both transport systems) and diluted 100-fold with medium containing no P_i exhibited high levels of phosphate uptake (Fig. 4B). We assume that this resulted from diluted [^{32}P]KH_2PO_4 in the medium that was still saturating the HATS and theoretically half-saturating the LATS. In contrast, after cells preloaded in the presence of 5 μM [^{32}P]KH_2PO_4 were diluted 100-fold with medium containing no P_i, neither the HATS in CIAT899 (Fig. 4A) nor the LATS in CAP45 (Fig. 4C) was saturated with respect to the solute substrate, and therefore the cells exhibited very reduced or no uptake activity.

Characterization of the transposon insertion site. A sequence analysis of the chromosomal DNA adjacent to the transposase end of Tn*5*B22 revealed a 151-bp segment immediately adjacent to Tn*5*B22 that exhibited 48 to 52% identity and 74 to 78% similarity to KdpE of *E. coli* (50), *Clostridium acetobutylicum* (45, 46), and *Mycobacterium tuberculosis* (11). KdpE is the cytoplasmic response regulator that is paired with the sensor kinase KdpD, and together these proteins govern expression of the high-affinity potassium transport system in response to changes in medium osmolarity or to potassium-limiting conditions.

DISCUSSION

Kinetic analysis showed that *R. tropici* CIAT899 has two P_i transport systems whose kinetic properties differ significantly. In contrast, CAP45 had a single P_i transport system that exhibited low affinity for P_i. At the solute substrate concentrations used in our assays, the apparent lack of transport activity via a HATS in this mutant allowed us to characterize the LATS. The kinetic properties of the *R. tropici* HATS suggest that it is not atypical. Its apparent K_m (0.45 μM) is very similar to the apparent K_m values reported for the HATS of *E. coli* (30), *P. aeruginosa* (26), and *A. johnsonii* (48). While it exhibited a V_{max} that is appreciably higher than the V_{max} values measured for the HATS of *E. coli* (30) and *P. aeruginosa* (26), it is very similar to the V_{max} observed for the HATS of *A. johnsonii* (48). The K_m of the LATS is much higher and indeed is more consistent with the range of values reported for secondary P_i transport systems in these bacteria (26, 48, 53).

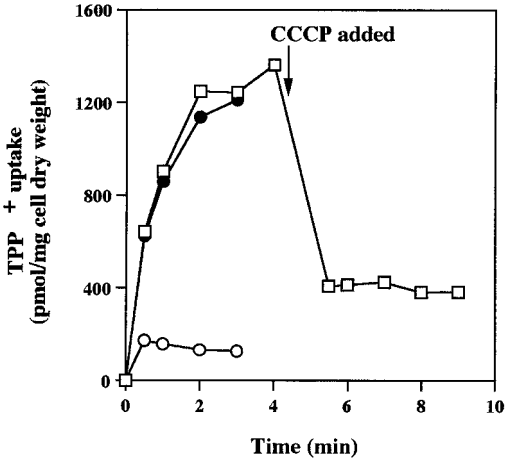

FIG. 3. Effect of CCCP on TPP$^+$ uptake and accumulation in *R. tropici* CIAT899. CCCP (5 μmol · 0.125 mg [dry weight]$^{-1}$ · ml^{-1}) was (○) or was not (●) added before [^{3}H]TPP$^+$ (18 μM; specific activity, 27.5 μCi · μmol^{-1}) was added. In other experiments (□), CIAT899 was allowed to take up [^{3}H]TPP$^+$ for 4 min, CCCP was added after 4.5 min, and then sampling commenced at 5 min. Cell samples were taken at the times shown and as described in Materials and Methods. The data show the typical effect of CCCP on [^{3}H]TPP$^+$ uptake and accumulation and are from one of the three independent assays performed.

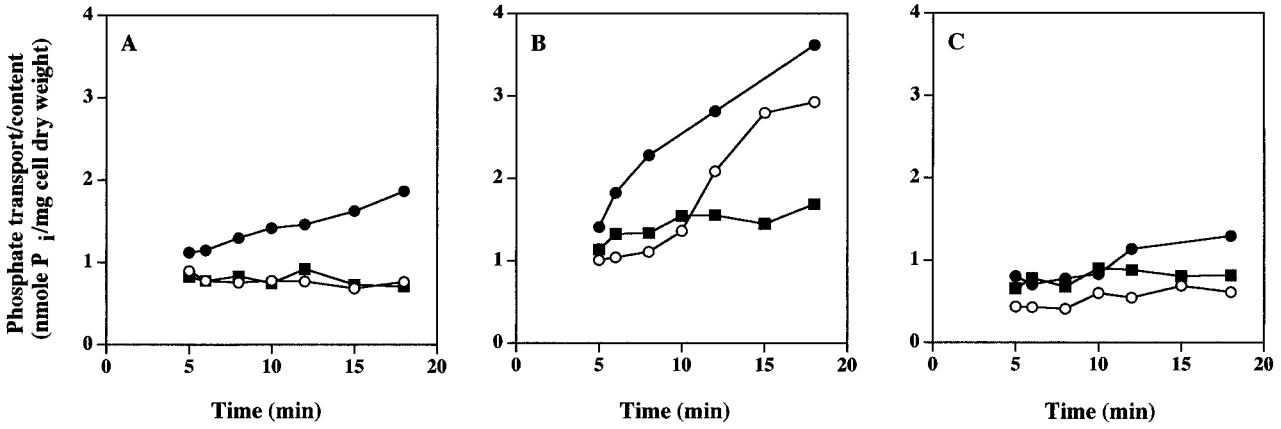

FIG. 4. Absence of P_i exchange and efflux in *R. tropici* CIAT899 and CAP45. (A) $-P_i$ cells of CIAT899 were loaded with 5 μM [^{32}P]KH_2PO_4 (specific activity, 22.5 μCi · μmol^{-1}) for 4 min and then diluted 100-fold with a medium containing 5 μM [^{32}P]KH_2PO_4. Symbols: ●, uptake control; ○, no P_i (efflux); ■, 25 μM unlabeled P_i (exchange). (B and C) $-P_i$ cells of CIAT899 (B) and CAP45 (C) were loaded with 400 μM [^{32}P]KH_2PO_4 (specific activity, 22.5 μCi · μmol^{-1}) for 4 min and then diluted 100-fold with a medium containing 400 μM [^{32}P]KH_2PO_4. Symbols: ●, uptake control; ○, no P_i (efflux); ■, 2 mM unlabeled P_i (exchange). The results are typical of the results of two experiments in which this response was documented.

Both P_i transport systems have characteristics that are consistent with ABC-type transporters (8). Both are shock sensitive, losing roughly 80% of their transport activity when periplasmic proteins are lost (Fig. 2 and Table 3). In addition, the ATPase inhibitor DCCD (Table 1) eliminated the transport activities of both systems. In contrast, the Δp dissipator CCCP, which has been shown to strongly inhibit secondary transport systems (7, 14), affected neither system (Table 1). Under the assay conditions used in routine P_i transport experiments, CCCP either significantly reduced TPP^+ uptake or caused the release of TPP^+ that had accumulated in response to an intact membrane potential (Fig. 3). Finally, the unidirectional uptake activity, as shown by the lack of apparent efflux and exchange activity observed with both systems (Fig. 4), also indicates that both the HATS and the LATS belong to the traffic ATPase class of solute transport systems. To summarize, the data obtained in this study suggest that *R. tropici* CIAT899 has two P_i transport systems. These transport systems differ in their affinities for P_i (Table 2) but are otherwise similar. Both are inducible by P_i limitation (Table 2), are shock sensitive (Fig. 2 and Tables 3), and utilize ATP to engage P_i transport (Table 1). The presence of two P_i transport systems in *R. tropici* is in contrast to the single P_i transport system reported for some rhizobia (41), and the presence of two functional traffic ATPase primary P_i transporters has not been reported for any of the other bacteria studied thus far (26, 34, 48, 54), including *S. meliloti* (49).

Additional, but indirect, evidence suggesting that at least the HATS of *R. tropici* is a multicomponent ABC type of solute transport system comes from the complex Pho phenotype of CAP45. In addition to the absence of a HATS, CAP45 also expresses alkaline phosphatase constitutively. These two traits also occur together in *E. coli* (12, 13, 52) and *S. meliloti* (5) mutants whose multicomponent HATS are affected. In both of the latter species, an operon arrangement is involved, and the operon typically includes genes coding for a periplasmic solute-binding protein, two integral membrane proteins, and an ATP-binding protein. Mutations in these operons result in a loss of the high affinity P_i transport function and also result in a loss of normal Pho regulation (i.e., constitutive expression of alkaline phosphatase, the marker enzyme for the P_i stress response). Analysis of the transposon insertion site in CAP45 revealed that the interrupted gene is *kdpE*. In both *E. coli* and *C. acetobutylicum* (46, 50), KdpE has been shown to be the cytoplasmic response regulator of a two-component regulatory pair which includes the sensor KdpD. Also in both of these bacteria, genes coding for KdpDE are arranged in an operon and are located immediately adjacent to the *kdp* operon, which codes for an ABC-type high-affinity K^+ transport system that is upregulated in response to low potassium concentrations in the medium or to low osmotic conditions (for reviews see references 3 and 38). In order to assess the effect (if any) of the affected region of the chromosome on the Pho and P_i transport phenotypes of CAP45, efforts to clone and fully characterize this region are currently under way and will be the subject of a subsequent report.

Functional duplication has been found previously in *R. tropici* (22, 32), and indeed reiteration is not uncommon in members of the *Rhizobiaceae* (18, 37). Therefore, the presence of two functional P_i stress-inducible P_i transport systems in CIAT899 is not without precedent. Two separate ABC type P_i transport operons that exhibit homology to the *E. coli pst* operon have been identified in the unrelated organism *M. tuberculosis* (10, 11, 27), and this finding implies that perhaps there are at least two traffic ATPase P_i transporters in mycobacteria (27). To our knowledge, these systems have not been characterized at the physiological level, and therefore it is not known if they are functional or to what extent they differ in their kinetic properties.

As discussed above, in broth culture *R. tropici* does not express alkaline phosphatase until the medium P_i concentration decreases to approximately 1 μM (1). The high levels of alkaline phosphatase in *R. tropici* bacteroids (1) suggest that under normal growth conditions the host plant perhaps distributes small amounts of P_i to the bacteroids and that the P_i concentration in the peribacteroid space may be quite low. Under such conditions, the HATS may be important to P_i acquisition by *R. tropici* bacteroids. Initial studies on the symbiotic properties of the mutant isolated in this study have shown that in situ P_i acquisition by CAP45 bacteroids is reduced during symbiosis and that the symbiotic competence of this mutant is also reduced (9).

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