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). To better understand phosphorus movement between the bacteroid and the host plant, P transport was characterized in *R. tropici*. We observed two P transport systems, a high-affinity system and a low-affinity system. To facilitate the study of these transport systems, a Tn5B22 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  and  values for the low-affinity system were estimated to be 34 ± 3 μM P and 118 ± 8 nmol of P·min⁻¹·mg (dry weight) of cells⁻¹, respectively, and the  and  values for the high-affinity system were 0.45 ± 0.01 μM P and 56 ± 5 nmol of P·min⁻¹·mg (dry weight) of cells⁻¹, respectively. Both systems were inducible by P 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 transport through both systems was eliminated by the ATPase inhibitor N,N'-dicyclohexylcarbodiimide; the P transport rate was correlated with the intracellular ATP concentration. Also, P 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 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*.

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 nodulation 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) 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 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-repressible high-affinity permease have been identified. In *E. coli*, the low-affinity P 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 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 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 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 conditions, the low-affinity Pit permease is expressed and is primarily responsible for P uptake. When *S. meliloti* is grown under P-limiting conditions, the Pit system is repressed, while the high-affinity PhoCDET system is induced and becomes the primary mechanism of P 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 assimilation and regulation in the microbial partner. As observed with other gram-negative bacteria (51), *R. tropici* induces alkaline phosphatase, and its P transport rate increases significantly in response to P limit-limiting conditions (1). The induction occurs when the medium P concentration is approximately 1 μM (1). *R. tropici* bacteroids isolated from...
nODULES OF BEAN PLANTS GROWN IN THE PRESENCE OF NONLIMITING PHOSPHORUS CONCENTRATIONS CONTAIN EXTREMELY HIGH LEVELS OF ALKALINE PHOSPHATASE, AS WELL AS A PStress-inducible acid phosphatase (1). THIS IMPLIES THAT UNDER NORMAL GROWTH CONDITIONS A BEAN PLANT PROVIDES VERY LOW LEVELS OF Pi TO THE BACTERIOIDS IN ITS NODULES. IN ORDER TO DETERMINE THE IMPORTANCE OF Pi SUPPLY FOR THE BEAN-BACTERIOPHAGE SYMBIOTIC SYSTEM, WE ARE NOW ASSESSING R. TROPICI Pi TRANSPORT SYSTEMS AND ESTIMATING THEIR KINETIC PROPERTIES. IN THIS REPORT, THE Pi TRANSPORT SYSTEMS OF R. TROPICI ARE DESCRIBED. LIKE S. MELLIOTI (49), THIS BACTERIUM HAS TWO DISTINCT Pi TRANSPORT SYSTEMS. HOWEVER, R. TROPICI APPEARS TO DIFFER FROM S. MELLIOTI AND ALL OTHER BACTERIA INVESTIGATED PREVIOUSLY SINCE BOTH Pi TRANSPORT SYSTEMS ARE INDUCIBLE BY Pi 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 Pi 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 1B (29), and CAP45 is a Pi transport mutant derived from CIAT899 (see below). CIAT899 was maintained on the minimal manitol-ammonium agar (MMNH4-OP) (43). The strain was grown under the same medium, except that β-galactosidase (GLP) replaced manitol as the sole carbon source and gentamicin was included at a final concentration of 25 mg · l−1. The other antibiotics used in the experiments were ampicillin (100 μg · ml−1) and tetracycline (25 mg · l−1). In experiments in which Pi-starved cells (−Pi cells) were used, the cells were incubated in MMNH4-OP which lacked added phosphorus but was buffered to pH 7.2 with 5 mM MES (morpholinopropanesulfonic acid) and 10 mM MOPS (morpholino-propansulfonic acid) (MMNH4-OP) (43).

Mutant isolation. Pho regulatory mutants that are constitutive for the PStress- inducible alkaline phosphatase in Escherichia coli (E. coli) were used to dissipate all components of the periplasmic space. EDTA treatment was used to dissociate the β-galactosidase mutation from the E. coli wild-type strain. Panel VI shows that chloramphenicol did not interfere with Pi transport (results not shown) but did inhibit the synthesis of alkaline phosphatase for at least 5 h. Thus, we concluded that deletion of the alkaline phosphatase gene from E. coli is not a prerequisite for the demonstration of a high-affinity Pi transport system.

Transport assays. Early-stationary-phase MMNH4 cultures were washed twice in MMNH4-OP and resuspended in MMNH4-OP to an optical density of 0.60 at an absorbance of 395 nm. To obtain Pi− cells, washed cells were incubated in MMNH4-OP at 30°C for 7 h in order to allow for maximum induction of Pi− transport (1). Chloramphenicol (50 mg · l−1) was added to stop further protein synthesis. Cells not starved for Pi+ (Pi+) cells were prepared in the same way except that chloramphenicol was added immediately after washing and the cells were used immediately. In preliminary experiments, we found that chloramphenicol did not interfere with Pi transport (results not shown) but did inhibit the synthesis of alkaline phosphatase for at least 5 h. Thus, we concluded that inorganic phosphate synthesis in Pi+ cells did not occur during the experiments performed with Pi− cells.

The standard transport assay was conducted in an orbital shaker water bath at 30°C. Washed cells were diluted with MMNH4-OP to a concentration of 0.025 mg · ml−1. In the first method of measurement, 0.125 mg (dry weight) of cells · ml−1 was used to test that the Pi transport activity was unchanged. After 5 min of preincubation in MMNH4-OP, the transport assay was initiated by adding P2Pi (at concentrations specified below) to [3H]Pi (final concentration, 1 μCi · ml−1). The [3H]Pi-containing solution was filtered prior to use in order to remove any extraneous particles that had adsorbed to the label. Cell samples (0.5 ml) were withdrawn at 20-s intervals (unless otherwise specified); each sample was collected on a 0.5-μm-pore-size glass fiber filter (Gelman Sciences, Ann Arbor, Mich.) and washed with 2 ml of 0.1-M Pi transport rinse buffer, which contained 20 mM MES and 5 mM KH2PO4 (pH 6.5). The filters were placed in counting vials, 20 ml of H2O 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 assays. Methods used by Packer and Rosenberg (30) were modified slightly for use with R. tropici. Briefly, −Pi cells were loaded for 4 min with [32P]KH2PO4 (either 5 or 400 μM; specific activity, 22.5 Ci · μmol−1) at 30°C and then diluted 100-fold with MMNH4-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.

Dynamic shock treatment. An osmotic shock method 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 MgSO4, at room temperature. The shock-treated cells were collected by centrifugation, gentamycin was added, and then Pi was transferred to MMNH4-OP, and then used for Pi 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 pellet 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 KH2PO4 (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+). −Pi 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 1 mM EDTA. At specific times and then collected and washed with 0.3-ml transport rinse buffer, which contained 20 mM MES and 5 mM KH2PO4 (pH 6.5). The filters were placed in counting vials, 20 ml of H2O 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.

Energy coupling. (i) Qualitative determination of membrane potential. Membrane potential was used to dissipate all components of the Δp (23, 25, 35). 400 μM, but without CCCP by using the membrane and conditions described above for Pi transport, except that choline chloride (150 μM) was added to the Δp assay system. Binding of TPP+ to anionic groups at the cell surface (28) but does not interfere with Pi uptake (data not shown).

For the TPP+ uptake assays we used MMNH4-OP with or without CCCP (final concentration, 1 μM). Cells (1 ml) were washed twice with 30 mM EDTA-treated −Pi 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 [3H]TPP+ (final concentration, 18 μM; specific activity, 27.5 Ci · μmol−1) was added. After [3H]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 Pi transport experiments. In the second type of experiment, [3H]TPP+ was added to initiate the transport assay, the cells were allowed to accumulate [3H]TPP+ for 4 min, and then CCCP was added for 4.5 min; this was followed by cell sampling. For both types of experiments, the [3H]TPP+ content of the cells was measured by placing the filters in counting vials, adding 20 ml of scintillation solution (Scintissile 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 Pi transport, Pi transport assays were performed as described above for the routine assays, except that the cells were incubated in the presence of CCCP (1 μM of CCCP per 0.025 mg [dry weight] of EDTA-treated −Pi cells per ml) at 30°C with [3H]TPP+ (specific activity, 27.5 Ci · 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. [3H]TPP+ was not included in the assay mixtures.

In experiments performed to determine the effect of CCCP on Pi transport, Pi transport assays were performed as described above for the routine assays, except that the cells were incubated in the presence of CCCP (1 μM of CCCP per 0.025 mg [dry weight] of EDTA-treated −Pi cells per ml) at 30°C with [3H]TPP+ (specific activity, 27.5 Ci · 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. [3H]TPP+ was not included in the assay mixtures.
was used to initiate sequencing from the transposase terminal (40).

Foster City, Calif.) and an ABI model 310 genetic analyzer (PE Applied Bio-

was sequenced by using an ABI Prism BigDye kit (PE Applied Biosystems, identical to the fragment in the genome of mutant CAP45. The flanking DNA

were analyzed by restriction analysis to verify that each contained a single cloned

gene). The ligation mixture was transformed into

E. coli

phosphatase specific activities of about 800 U (1 U obtained from these mutants revealed that they had alkaline

alkaline phosphatase. Enzyme assays of periplasmic extracts

mutagenesis of

studied. To determine ATP concentrations, the reaction mixtures used were

(i.e., we avoided reductions in the ATP pool size via loss of H

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% [v/v]; 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

transport assay reaction mixtures, except that no radioligand 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

were added. After incubation (see below), cellular ATP was extracted as described by

were analyzed by restriction analysis to verify that each contained a single cloned

were treated with EDTA as described in the text, preincubated with an inhibitor and then assayed to determine P

transport rates, and therefore an ethanol control was included in all experiments.

The values in parentheses are percentages of reduction in P

transport compared to the controls.

The protocols of Sambrook et al. (36) were used

and the balance of the transposon and flanking chromosomal DNA. The

transposase portion of Tn

insertion site in the mutant was characterized by selectively subcloning the

Isolation of phosphate transport mutant CAP45. Tn5B22

samples was calculated by using the following equation: ATP concentration

by Joshi et al. (23). ATP concentrations were determined by using the luciferase

was added. After incubation (see below), cellular ATP was extracted as described by

ensures that two separate transport systems were present. Eadie-Hof-

sequent mutants that had reduced P

transport rates. Southern blot analysis of chromosomal DNA prepared from the P

transport mutants verified that Tn5B22 was present, and all of the blot

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

transport in both +P

cells and −P

cells of CIAT899 revealed that two separate transport systems were present. Eadie-Hof-

ste plots of P

transport in −P

cells of CIAT899 and CAP45 are shown in Fig. 1. As measured at P

concentrations of 0.1 to 500 μM and calculated from a linear regression analysis, the estimated K

values for two transport systems differed by approximately 2 orders of magnitude. In addition to being ex-

pressed under high-P

growth conditions, both systems were

induced in response to P

deprivation, as shown by the in-

creases in the V

max values of −P

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-

phatase activity of CIAT899 −P

cells is approximately 30 U, and the alkaline phosphatase activity of CIAT899 −P

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

transport phenotype we identified isolates that had reduced P

transport rates. Southern blot analysis of chromosomal DNA prepared from the P

transport mutants verified that Tn5B22 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.

Isolation of phosphate transport mutant CAP45. Tn5B22

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 alkali- nophosphate. 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

cells were used. Typically, the alkaline phos-

TABLE 1. Effect of Δp dissipation and ATP depletion on P

uptake in R. tropici CIAT899 and CAP45

| Treatment         | Strain     | P

uptake (nmol of P

/min·mg [dry wt] of cells) | ATP concn (nmol of ATP/mg [dry wt] of cells) |
<table>
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<tr>
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<tbody>
<tr>
<td>Ethanol control</td>
<td>CIAT899</td>
<td>26.7 ± 3.2</td>
<td>41.8 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>CAP45</td>
<td>2.4 ± 0.3</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>CCCP (1 μM)</td>
<td>CIAT899</td>
<td>23.8 ± 1.4 (11)</td>
<td>44.9 ± 2.1 (0)</td>
</tr>
<tr>
<td></td>
<td>CAP45</td>
<td>2.1 ± 0.1 (12)</td>
<td>12.2 ± 0.1 (0)</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>CIAT899</td>
<td>10.2 ± 2.2</td>
<td>11.76 ± 1.97</td>
</tr>
<tr>
<td></td>
<td>CAP45</td>
<td>2.2 ± 0.1</td>
<td>7.06 ± 0.37</td>
</tr>
<tr>
<td>DCCD (100 μM)</td>
<td>CIAT899</td>
<td>0 ± 0 (100)</td>
<td>0.43 ± 0.08 (96)</td>
</tr>
<tr>
<td></td>
<td>CAP45</td>
<td>0.034 ± 0.001 (98)</td>
<td>0.29 ± 0.02 (96)</td>
</tr>
</tbody>
</table>

a −P

cells were treated with EDTA as described in the text, preincubated with an inhibitor and then assayed to determine P

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

transport or ATP concentration compared to the controls.

TABLE 2. Kinetic parameters of P

uptake in R. tropici CIAT899 and CAP45

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth conditions</th>
<th>High-affinity uptake</th>
<th>Low-affinity uptake</th>
</tr>
</thead>
</table>
|            | K

m (μM P

) | V

max (nmol of P

/min·mg [dry wt] of cells) | K

m (μM P

) | V

max (nmol of P

/min·mg [dry wt] of cells) |
| CIAT899    | −P                | 0.45 ± 0.01          | 86.2 ± 4.9          | 9.6 ± 1.0          | 153.8 ± 13.6       |
|           | +P                | 0.34 ± 0.02          | 0.22 ± 0.01         | 35.7 ± 1.0         | 1.33 ± 0.01        |
| CAP45      | −P                | ND                   | ND                  | 34.3 ± 2.7         | 118.0 ± 7.5        |
|           | +P                | ND                   | ND                  | 33.1 ± 1.3         | 40.1 ± 0.02        |

a The kinetics of P

uptake were analyzed by using Eadie-Hofstee plots. The initial velocities in +P

and −P

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

concentration was replicated three times in each experiment.

b ND, not detected.
all \( P_i \) transport behavior. The \( K_m \) for this \( P_i \) permease was found to be 34 \( \mu \)M, 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 \( \mu \)M 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 non-shocked 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 \( \Delta \phi \) 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 \( \Delta \phi \) 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 (\( \Delta \psi \)) (25), which in cowpea rhizobia has been shown to be unaffected by changes in pH (20).
The effects of CCCP on $\Delta p$, as measured by uptake and accumulation of the $\Delta p$ probe $[^3H]TPP^*$, are shown in Fig. 3. In one set of experiments, CCCP was included in each cell suspension before $[^3H]TPP^*$ was added during the uptake assay (Fig. 3A). These experiments showed that CCCP dissipated $\Delta p$, which resulted in significantly reduced $[^3H]TPP^*$ uptake and accumulation. In other experiments, CCCP was added to cells that were in the process of accumulating $[^3H]TPP^*$. This addition resulted in the immediate release of $[^3H]TPP^*$; again, the data showed that CCCP treatment dissipated a significant portion of the $\Delta p$ (interior negative) but also demonstrated that $[^3H]TPP^*$ did not simply bind to cell components, as it was readily released when the $\Delta p$ was dissipated. On the basis of several such experiments in which CCCP treatment consistently either resulted in the release of $[^3H]TPP^*$ or inhibited $[^3H]TPP^*$ uptake and accumulation by 50 to 90% compared to control cells, we concluded that CCCP largely eliminated the $\Delta p$ and could be used to assess the importance of the $\Delta p$ 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 $\Delta p$), 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 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 $\Delta p$ 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 $\mu$M $[^32P]KH_2PO_4$ (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 $[^32P]KH_2PO_4$ 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 $\mu$M $[^32P]KH_2PO_4$ 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 Tn5B22 revealed a 151-bp segment immediately adjacent to Tn5B22 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 osmolality 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 $\mu$M) 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).
Both Pi 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 activity 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 Pi 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 Pi transport systems. These transport systems differ in their affinities for Pi (Table 2) but are otherwise similar. Both are inducible by Pi limitation (Table 2), are shock sensitive (Fig. 2 and Table 3), and utilize ATP to engage Pi transport (Table 1). The presence of two Pi transport systems in R. tropici is in contrast to the single Pi transport system reported for some rhizobia (41), and the presence of two functional traffic ATPase primary Pi 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. melliloti (5) mutants whose multicompartment 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 Pi transport function and also result in a loss of normal Pho regulation (i.e., constitutive expression of alkaline phosphatase, the marker enzyme for the Pi 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 Pi 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 Pi stress-inducible Pi transport systems in CIAT899 is not without precedent. Two separate ABC type Pi 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 functional Pi 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 Pi 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 Pi to the bacteroids and that the Pi concentration in the peribacteroid space may be quite low. Under such conditions, the HATS may be important to Pi acquisition by R. tropici bacteroids. Initial studies on the symbiotic properties of the mutant isolated in this study have shown that in situ Pi acquisition by CAP45 bacteroids is reduced during symbiosis and that the symbiotic competence of this mutant is also reduced (9).

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*Escherichia coli* phosphate-specific transport system, a member of the traffic 
ATPase (or ABC) family of membrane transporters. A role for proline 
