



Phosphate transport in *Rhizobium tropici* under free-living and symbiosis conditions
by Lina Maria Botero

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Soils
Montana State University

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Abstract:

Phosphorus is one of the several factors affecting nitrogen fixation and is limiting to crop production. Plants dependent on symbiotic nitrogen have a higher requirement for phosphorus than plants using mineral nitrogen but little is known about the basis for this response. Experiments were conducted to determine how many transport systems for inorganic phosphorus (Pi) are present in *Rhizobium tropici* and to understand what role these transport systems play during symbiosis under conditions of adequate or limiting Pi. The transport of Pi was characterized in free-living cells of the wild-type strain CIAT899. Two transport systems with high- and low-affinity for Pi are present under sufficient and limiting Pi growth conditions which is in contrast to a single Pi transport system reported for other rhizobia. A Pi transport mutant (CAP45) deficient in transport activity via the high-affinity system allowed for the characterization of the low-affinity system in the absence of the other system. The transport systems in *R. tropici* differ in affinity for Pi by two orders of magnitude, are inducible by Pi limitation, are shock-sensitive, and apparently utilize ATP, or a derivative of ATP, to energize the transport process. The occurrence of two Pi transport systems having properties of traffic ATPases, periplasmic-binding protein-dependent primary transporters, appears to be novel relative to other bacteria thus far studied. The symbiosis phenotype of the mutant CAP45 was assessed using bean plants grown in growth pouches containing either a complete nutrient solution or a nutrient solution without Pi. The high-affinity Pi transport system seems to be required for a fully effective symbiosis since plants nodulated with the mutant strain showed significant reduction in plant dry matter. ³²P tracer studies showed that in situ rates of Pi accumulation were significantly lower in the mutant regardless of the Pi status of the plants. The decrease in Pi accumulation in bacteroids from plants nodulated by the mutant strain suggests that a mechanism involving the bacteroid is in place to enhance Pi uptake. Furthermore, these findings indicate that inorganic phosphorus is an important source of phosphorus for the bacteroids.

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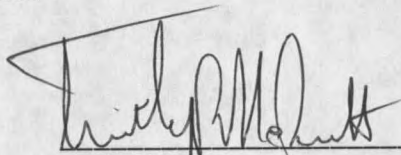
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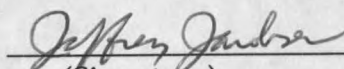
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TABLE OF CONTENTS

	page
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	ix
CHAPTER 1. INTRODUCTION	1
References Cited	5
CHAPTER 2. LITERATURE REVIEW	6
Phosphorus Metabolism in Rhizobiaceae	6
Gene Regulation by Phosphate in <i>Escherichia coli</i>	6
Phosphorus Physiology in the Rhizobiaceae	10
Effects of Phosphorus Supply on Symbiosis	12
Phosphorus Sources for Bacteroids	14
Solute Transport in Bacteria	15
Passive Transport Across the Cell Membrane	16
Active Transport	17
The Chemiosmotic Concept	17
Ionophores	20
Uniport, Symport, and Antiport	21
ATP-Coupled Solute Transport Systems	23
H ⁺ -Translocating ATPase	23
P-Type ATPases	24
Transport Systems Utilizing Periplasmic Binding Proteins ..	25
Phosphate Transport Systems	29
Pit System (Phosphate Inorganic Transport)	30
Pst System (Phosphate Specific Transport)	31
Organophosphate Uptake	32
Summary of Transport of Phosphate and Phosphorylated Compounds in <i>Escherichia coli</i>	33
References Cited	34

TABLE OF CONTENTS-Continued

CHAPTER 3. <i>Rhizobium tropici</i> HAS TWO PHOSPHATE-REPRESSIBLE PHOSPHATE TRANSPORT SYSTEMS	40
Introduction	40
Materials and Methods	42
Strains and Culture Conditions	42
Transport Assays	44
Phosphate Exchange and Efflux	45
Osmotic Shock Treatment	45
EDTA Treatment of Cells	46
Determination of Membrane Potential ($\Delta\Psi$)	47
ATP Determination	48
Results	49
Isolation of the Phosphate Transport Mutant CAP45	49
Kinetic Parameters of Phosphate Uptake	50
Effect of Osmotic Shock on Phosphate Uptake	53
Energy Coupling to Phosphate Transport	56
Exchange and Efflux of Phosphate	60
Discussion	60
References Cited	65
CHAPTER 4. ROLE OF THE HIGH AFFINITY PHOSPHATE TRANSPORTER IN <i>Rhizobium tropici</i> DURING SYMBIOSIS	69
Introduction	69
Materials and Methods	71
Strains	71
Phosphate Uptake Assay	71
Plant Culture and Symbiosis Studies	71
Bacteroid ^{32}P Accumulation <i>in situ</i>	74
Results	76
Free-living <i>Rhizobium tropici</i>	76
Symbiosis Studies	76
Bacteroid ^{32}P Accumulation	81
Discussion	81
References Cited	89
CHAPTER 5. THESIS SUMMARY AND CONCLUSIONS	92

LIST OF TABLES

Table	page
1. Transport systems of inorganic phosphorus, Glycerol-3-Phosphate, and hexoses phosphates in <i>Escherichia coli</i>	34
2. Kinetic parameters of P_i uptake in <i>R. tropici</i> CIAT899 and CAP45	52
3. Kinetic parameters of P_i uptake in osmotic shock-treated cells	55
4. Release of periplasmic proteins from CIAT899 by osmotic shock treatment	56
5. Effect of proton motive force dissipation and ATP depletion on P_i transport in <i>R. tropici</i> CIAT899 and CAP45	59
6. Symbiosis phenotype of <i>R. tropici</i> wild-type strain CIAT899 and the high-affinity P_i transport mutant CAP45	80

LIST OF FIGURES

Figure	page
1. Solute transport in bacteria	19
2. Eadie-Hofstee plots of initial P_i uptake velocities in <i>R. tropici</i> CIAT899 and CAP45	51
3. Effect of osmotic shock on the uptake of P_i in $-P_i$ cells of <i>R. tropici</i> CIAT899 and CAP45	54
4. Effect of CCCP on TPP^+ uptake in <i>R. tropici</i> CIAT899	58
5. Absence of P_i exchange and efflux in <i>R. tropici</i> CIAT899 and CAP45	61
6. Phosphate uptake in <i>R. tropici</i> CIAT899 and CAP45	77
7. Effect of different strength nutrient solution on shoot dry weight, salt accumulation and nodule dry weight	79
8. <i>In situ</i> CIAT899 and CAP45 bacteroid P_i accumulation	82

ABSTRACT

Phosphorus is one of the several factors affecting nitrogen fixation and is limiting to crop production. Plants dependent on symbiotic nitrogen have a higher requirement for phosphorus than plants using mineral nitrogen but little is known about the basis for this response. Experiments were conducted to determine how many transport systems for inorganic phosphorus (P_i) are present in *Rhizobium tropici* and to understand what role these transport systems play during symbiosis under conditions of adequate or limiting P_i . The transport of P_i was characterized in free-living cells of the wild-type strain CIAT899. Two transport systems with high- and low-affinity for P_i are present under sufficient and limiting P_i growth conditions which is in contrast to a single P_i transport system reported for other rhizobia. A P_i transport mutant (CAP45) deficient in transport activity via the high-affinity system allowed for the characterization of the low-affinity system in the absence of the other system. The transport systems in *R. tropici* differ in affinity for P_i by two orders of magnitude, are inducible by P_i limitation, are shock-sensitive, and apparently utilize ATP, or a derivative of ATP, to energize the transport process. The occurrence of two P_i transport systems having properties of traffic ATPases, periplasmic-binding protein-dependent primary transporters, appears to be novel relative to other bacteria thus far studied. The symbiosis phenotype of the mutant CAP45 was assessed using bean plants grown in growth pouches containing either a complete nutrient solution or a nutrient solution without P_i . The high-affinity P_i transport system seems to be required for a fully effective symbiosis since plants nodulated with the mutant strain showed significant reduction in plant dry matter. ^{32}P tracer studies showed that *in situ* rates of P_i accumulation were significantly lower in the mutant regardless of the P_i status of the plants. The decrease in P_i accumulation in bacteroids from plants nodulated by the mutant strain suggests that a mechanism involving the bacteroid is in place to enhance P_i uptake. Furthermore, these findings indicate that inorganic phosphorus is an important source of phosphorus for the bacteroids.

CHAPTER 1

INTRODUCTION

Legume nitrogen fixation occurs as a result of a symbiotic association between a plant and a bacterium. Both organisms participate in a complex biochemical communication that culminates in the formation of root nodules. These nodules function as a specialized plant organ to provide a protective environment where the biochemical exchange between the symbionts continues. In the mature functioning nodule, the plant provides the bacteria with organic acids derived from photosynthetic sugars, which they use as an energy source for nitrogen fixation. Nitrogen fixation is an energy intense process where atmospheric N_2 gas is reduced to NH_4^+ , a form of nitrogen that all organisms can use. In simplest terms, this symbiosis revolves around the exchange of reduced carbon (photosynthesis) for reduced nitrogen (nitrogen fixation).

The ability of legumes to grow without the application of nitrogen fertilizers has been utilized for hundreds of years. Some legumes are grown for their seed, which are used as a human dietary source of protein or cooking oil, while other legumes are used as forage crops. In early times, it was

recognized that legumes tend to "sweeten" the soil, and that other crops such as corn or wheat grew much better in fields previously planted to a legume. Such practice represents the earliest application of a technique known as crop rotation, and while there are other benefits derived from crop rotations, the green manure function of legumes makes them an important part of modern agriculture. Currently, agricultural producers are increasingly moving towards "sustainable" agricultural systems. Such cropping systems involve techniques that utilize fewer fertilizer and herbicide inputs, and are in response to society's recognition that "soil health" needs to be nurtured and because consumers are more concerned about chemical additives used in food production. Because of their ability to grow without nitrogen fertilizer, legumes play an important role in crop rotations that are crucial to these sustainable systems.

Research within the past decade has made tremendous strides in understanding how nodules are formed, and how carbon and nitrogen are exchanged. However, there are many other aspects of the symbiosis that have not yet received the attention they deserve. A prominent example is that of phosphorus metabolism during symbiosis. Phosphorus is one of the several factors that affect nitrogen fixation and along with nitrogen, is a principal yield-limiting nutrient in many areas of the world. This is evident for legumes where significant responses to inorganic phosphorus (P_i) fertilizer are well documented (Graham and Rosas, 1979; Pereira and Bliss, 1987).

Virtually every aspect of this symbiosis is enhanced by P_i fertilizer application, and studies have shown that plants dependent on symbiotic nitrogen fixation have a higher P_i requirement for optimal growth than plants supplied with fertilizer nitrogen (Israel, 1987); of particular importance, is the enhanced rates of nitrogen fixation that results from P_i application. Our knowledge of phosphorus metabolism in this symbiosis, however, is mostly limited to a few studies documenting such crop responses and where some very basic bacterial responses to P_i starvation have been monitored. Therefore, it is clear that regardless of how much progress is made in understanding and engineering both partners for high rates of carbon and nitrogen exchange, the agronomic potential of this symbiosis will always be limited by phosphorus availability. Given our ignorance on what is obviously an important macronutrient, additional research aimed at understanding phosphorus exchange in this plant-microbe interaction is urgently needed.

Little is known about phosphorus exchange in this symbiosis. How is phosphorus provided to the bacteroids, as the inorganic ion or as an organic form? Does the host buffer the bacteroids from phosphorus shortage? Have bacteroids developed special mechanisms to deal with phosphorus stress conditions? It has been proposed that the growth of rhizobia in phosphorus limited environments is either controlled by the ability to attain a critical internal phosphorus level (Smart *et al.*, 1984), is related to the amount of storage phosphorus (Beck and Munns, 1984; Smart *et al.*, 1984) or is related to

the efficiency of phosphorus uptake and later utilization (Beck and Munns, 1984).

In the next chapter, a literature review begins with a summary of work accomplished on phosphorus metabolism in the Rhizobiaceae and is followed by a discussion of phosphorus nutrition in legumes and the effects of phosphorus on the *Bradyrhizobium/Rhizobium*-legume symbiosis. Then, a general overview of solute transport in bacteria, the mechanisms involved, and how the process is energized is provided. Because this thesis research focuses on phosphorus nutrition in *Rhizobium tropici* and the symbiosis it forms with bean (*Phaseolus vulgaris*), transport mechanisms specific to the acquisition of phosphorus receive special treatment.

The literature review is followed by chapters 3 and 4, which describe the research component of this thesis, which focus on mechanisms of inorganic phosphorus (P_i) transport in *Rhizobium tropici*. A mutant exhibiting a P_i transport phenotype was compared against the wild type strain in a variety of kinetic and inhibitor studies designed to determine how many transport systems are used, under what conditions they are employed, and the extent of their use during symbiosis. Chapters 3 and 4 are presented in a fashion similar to how they will be submitted for publication.

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CHAPTER 2

LITERATURE REVIEW

Phosphorus Metabolism in the Rhizobiaceae

Phosphorus is present in nucleic acids, phospholipids, and lipopolysacharides, and participates in many regulatory functions. While little is known about phosphorus metabolism and its regulation in rhizobia, enormous progress has been made in our understanding of phosphorus metabolism in enteric bacteria (see Torriani, (1990) and Wanner, (1993) for reviews). Therefore, for the purpose of providing a framework for considering phosphorus metabolism in rhizobia, *Escherichia coli* will be considered as a model.

Gene Regulation by Phosphate in *Escherichia coli*

The phosphate regulon (PHO) is comprised of at least 30 genes arranged in eight operons. These genes are all up-regulated when P_i becomes limiting, resulting in up to an 100-fold increase in protein expression. The best studied components of the PHO regulon include alkaline phosphatase (AP, encoded by *phoA*), genes expressed for phosphonate metabolism

(*phnCDEFGHIJKLMNOP*), genes involved in P_i transport (*pstABCphoU* operon), and the PhoE porin (Wanner, 1996). PHO regulation involves PhoR, PhoB and PhoU, as well as other gene products that have been shown to participate. The primary circuitry governing expression of *pho* genes begins with the two-component regulatory pair PhoR and PhoB; PhoR is the sensor and PhoB is the response regulator. PhoR autophosphorylates at the C-terminal and then behaves as a kinase to phosphorylate PhoB. When phosphorylated, PhoB activates transcription by binding to an 18-base consensus PHO box preceding all genes or operons within the PHO regulon. When P_i is in excess, it is suggested that the N-terminal portion of PhoR inactivates PhoB by dephosphorylation. This was inferred from experiments with PhoR⁻ mutants with various deletions at the 5' end which lacked the negative function (Torriani, 1990).

phoU is part of the *pst* (phosphate specific transport) operon. It encodes a small protein (27 KDa) whose function has not yet been defined. Mutant *phoU35* carries a missense mutation (the sequence of amino acids in the protein has changed) that results in the constitutive expression of *phoA*, but it displays wild type P_i transport (Torriani, 1990). A *phoU* deletion mutant (a region of DNA has been removed) also produces high levels of AP when grown in excess of P_i (Muda *et al.*, 1992). High copy number expression of *phoU* in cells grown with excess P_i does not affect AP regulation, implying that PhoU by itself has no negative effect on AP synthesis (Muda *et al.*, 1992).

It has been suggested that PhoU causes repression of AP synthesis by assuming a particular structure that activates PhoR phosphatase activity, and that PhoU has a role in P_i transport since a 80% decrease in the V_{max} for P_i uptake was observed in the *phoU* deletion mutant (Muda *et al.*, 1992). However, Steed and Wanner (1993) constructed *E. coli* mutants with defined deletions of the *pst-phoU* operon, and found that *phoU* mutations had no role in P_i transport but had a severe growth defect. They proposed that the PhoU protein has a role in the PHO regulon in addition to its role as a negative regulator, perhaps as an enzyme or a regulatory subunit of an enzyme for ATP synthesis.

Torriani and Rothman, (1961) isolated constitutive AP mutants by selecting for mutants capable of growing on β -glycerol phosphate (BGP) as a sole carbon source under conditions of excess P_i . To be taken up for use as a carbon source, BGP must first be dephosphorylated. In the presence of high inorganic phosphorus, AP synthesis is normally repressed, resulting in the inability to use BGP as a carbon source. Only mutants expressing AP under high P_i conditions would be capable of using BGP as a carbon source. The constitutive AP mutants isolated by Torriani and Rothman (1961) were grouped into two classes that mapped to two locations. One class were composed of *phoR* mutants and produced a low level of AP. The other class of mutants mapped to the *pst* operon and resulted in a high level of constitutive AP activity.

Pst⁻ mutants are altered in both P_i transport and normal regulation of *phoA*, but the defective P_i transport phenotype is not the cause of the AP constitutivity (Cox *et al.*, 1988; 1989). Missense mutations in the PstA or PstC proteins result in loss of phosphate transport through the *pst* system, but AP expression in these mutants remains sensitive to P_i levels in the medium. However, a missense change in the nucleotide binding domain of the PstB permease abolished both, AP expression and P_i transport. These results suggested that the PHO regulon is not controlled by the movement of P_i through the Pst system but the putative nucleotide binding site of the PstB is apparently required for both transport and repression of *pho* genes.

It should also be noted that two P_i-independent controls have been described (Wanner, 1993). They are both regulated by carbon source and appear to be coupled with subsequent steps in P_i metabolism. One is induced by glucose and requires the sensor CreC (formerly called PhoM), which will phosphorylate PhoB in response to an unknown signal for the incorporation of P_i into ATP via glycolysis, the tricarboxylic acid cycle, or ATP synthetase (Wanner, 1993). The other control is induced during growth on pyruvate and involves acetyl phosphate synthesis. Acetyl phosphate acts as an apparent nonspecific phospho-donor for response regulator proteins such as PhoB (Lukat *et al.*, 1992). These two controls are separate since mutations affecting CreC-dependent control do not affect acetyl-phosphate dependent control and vice versa. Also, mutations in genes that affect central metabolism alter these

two P_i -independent controls. These genes include ones for aerobic respiratory control (*arcA*), adenylate cyclase (*cya*), cAMP receptor protein (*crp*), isocitrate dehydrogenase (*icd*), malate dehydrogenase (*mdh*), exopolysaccharide production (*ops*), and others (Wanner, 1993). Cross regulation (controlling the activity of the response regulator by a nonpartner sensor) of the PHO regulon by CreC and acetyl phosphate are examples indicating that a link between different regulatory systems exists to coordinate cell metabolism (Wanner, 1992).

PhoE is an outer membrane protein that provides a channel for phosphorylated compounds to diffuse into the periplasmic space. Although PhoE synthesis is activated under conditions of P_i limitation and is subjected to PHO regulon control, PhoE is considered a nonspecific porin. It has only a slight preference for anions and therefore is not considered a 'phosphoporin' (Wanner, 1996).

Phosphorus Physiology in the Rhizobiaceae

Rhizobia vary in their external growth requirements for phosphate. The ability to store P_i as polyphosphate (poly-P) and utilize it for subsequent growth is strain dependent (Beck and Munns, 1984). Storage of poly-P may be a mechanism for supporting growth in P_i -free medium since it has been demonstrated that strains grown at high P_i levels were able to store sufficient P_i to allow several generations of growth after transfer into P_i -depleted medium (Beck and Munns, 1984; Smart *et al.*, 1984a).

Smart *et al.* (1984a) studied the growth responses of some rhizobial species with respect to P_i . Fast-growing cowpea (*Vigna unguiculata*) *Rhizobium* and *R. trifolii* strains incubated under P_i -rich conditions showed higher levels of total biomass phosphorus and poly-P compared with P_i -limited cells. In contrast, the P_i -status for the slow-growing *Bradyrhizobium* strain of soybean (*Glycine max*) did not affect either of these parameters. They also reported that P_i nutrition of snake bean bacteroids was relatively unaffected by the P_i status of the plant. Bacteroids contained high levels of stored phosphate even when they were under P_i -limited conditions and they showed low AP activity in both P_i -limited and P_i -rich plants. In a subsequent study, Smart *et al.* (1984b) investigated the response of free-living rhizobia to P_i -limiting conditions and found that both AP activity and P_i uptake were derepressed. In their study, P_i -limited cells of four species of *Rhizobium* and three species of *Bradyrhizobium* took up phosphate 10 to 180-fold that of P_i -rich cells, with K_m values ranging from 1.6 μM to 6 μM . However, AP activity was detected only in the *Rhizobium* species. Arsenate almost eliminated P_i uptake in P_i -limited cells, and slightly inhibited it in P_i -rich cells. The authors concluded that rhizobia, unlike *E. coli*, contain only a single energy-dependent system for the uptake of P_i . They also reported some aspects of bacteroid P_i metabolism. They found that P_i uptake was slightly higher in bacteroids from P_i -deficient plants than in those from P_i -sufficient plants, but the P_i uptake rate was only 5% of the rate observed with free-living P_i -limited

cells. Furthermore, the apparent K_m (7 μM) for phosphate uptake in these bacteroids was higher than the K_m (4 μM) obtained in free-living cells of snake bean (*Vigna unguiculata* s. sp. *sesquipedalis*).

Al-Niemi *et al.* (submitted) studied the regulation of the P_i -stress response in *R. meliloti* and found that AP was derepressed and P_i transport rates increased significantly when P_i levels in the growth medium decreased to approximately 10 μM . An *R. meliloti* PhoB (positive regulator of the PHO regulon) mutant isolated in their study did not induce for AP or increased rates of P_i transport when starved for P_i , but was able to fix nitrogen (Fix^+) on alfalfa (*Medicago sativa*) plants cultured under adequate and limiting P_i conditions. The lack of a Fix^- phenotype suggests that loss of a functional PhoB does not disturb symbiotic function of *R. meliloti*. However it is also possible that other regulator contributes to regulation of the PHO regulon of *R. meliloti* in the absence of PhoB.

Effects of Phosphorus Supply on Symbiosis

Studies have shown that symbiotically-grown legumes have a significantly greater P_i requirement than legumes provided with fertilizer nitrogen (Israel, 1987). This is an important observation as legumes are most commonly grown symbiotically and in many areas of the world, P_i is often limiting to crop production. A number of studies have demonstrated that almost every aspect of the symbiosis is enhanced by P_i fertilizer application

(Graham and Rosas, 1979; Pereira and Bliss, 1987). For example, whole plant nitrogen concentration, plant dry matter, nodule number, nodule mass and nitrogenase activity are increased by phosphorus fertilization. Distribution of phosphorus within the symbiosis is not always proportionate, for example, bean (*Phaseolus vulgaris*) nodules contain about 12% of total plant phosphorus, while accounting for only 6% of total plant dry matter (Pereira and Bliss, 1989). Phosphorus fertilization of field-grown common beans results in a 12-fold increase in nodule phosphorus content as compared to 6-fold increase in leaf and stem, and a 2-fold increase in roots (Graham and Rosas, 1979).

Sa and Israel (1991) studied the effects of long term phosphorus deficiency in soybean nodules. They found that adenylate levels, and ATP concentrations in the plant cell fraction of the nodule were decreased in response to phosphorus limitation, whereas these parameters were not affected in the bacteroid fraction of the nodule. These observations indicated that phosphorus deficiency impaired energy transducing pathways, such as oxidative phosphorylation in the plant cell fraction of the nodules to a much greater extent than in the bacteroids. On the other hand, the bacteroid dry mass per unit nodule dry mass, bacteroid nitrogen concentration, and the specific nitrogenase activity were significantly reduced by phosphorus deprivation. A correlation between decreased specific nitrogenase activity and decreased ATP levels in the plant cell fraction of the nodule was found.

Therefore, Sa and Israel (1991) suggested that phosphorus deficiency decreases the specific nitrogenase activity by inhibiting one or more energy-dependent reactions in the plant cell fraction of the nodules.

Phosphorus Sources for Bacteroids

To date, little is known about the kinds and amount of phosphorus exchange that occurs between the symbionts. For example, is phosphorus provided to the bacteroids as the inorganic ion or in an organic form? A recent study suggests that inorganic phosphorus is an important source of phosphorus for *Rhizobium* bacteroids (Bardin *et al.*, 1996). The authors reported that in an *R. meliloti* mutant previously referred to as *ndvF* due to its defect in nodulation (Charles *et al.*, 1991), the *ndvF* locus contains four genes, *phoCDET*, which encode a binding protein-dependent P_i transport system. They suggested that the impaired symbiosis phenotype of this bacterium results from its failure to utilize P_i for growth during the nodule infection process. However, it is not known if phosphorus is first presented to the bacteroids as an organic form, which would first require phosphatase activity to release P_i for uptake through the P_i transporter.

Several observations suggest the involvement of organic forms of phosphorus as sources for bacteroids. There may be several organic-phosphorus sources for bacteroids, including the phosphorylated derivatives of inositol. Phosphatidylinositol is a major phospholipid in plant plasma membranes (Drobak, 1993), and a recent study has shown that a glycosylated

form of an inositol-containing phospholipid is found in the plant-derived peribacteroid membrane that surrounds bacteroids in infected pea (*Pisum sativum*) cells (Perotto *et al.*, 1995). Secondly, very high levels of a phosphate-repressible AP and an acid phosphatase have been found in *R. tropici* bacteroids (Al-Niemi *et al.*, 1997). The expression of these enzymes suggests that very low concentrations of P_i exist in the symbiosome (unit comprised of the peribacteroid membrane and the enclosed bacteroids) and that phosphatases may be required for P_i acquisition. Furthermore, acid phosphatases have been reported in the periplasm of *Bradyrhizobium japonicum* bacteroids (Kinnback and Werner, 1991).

Solute Transport in Bacteria

Growth and survival of bacteria depends on their ability to obtain essential nutrients from the environment. Nutrient acquisition involves some type of transport mechanism whereby the solute is moved across a membrane that separates the cell from the environment. In Gram negative bacteria, the solute must first pass through the outer membrane, which acts as molecular sieve, excluding molecules greater than 800 molecular weight. Otherwise, this membrane exerts little selection.

By contrast, the cytoplasmic membrane of both Gram positive and Gram negative bacteria is quite specific. The hydrophobic nature of the cytoplasmic membrane allows it to function as a tight barrier so that the

passive movement of polar solutes does not readily occur. Solute movement across this membrane requires specific transport mechanisms and the expenditure of energy. Some small, relatively hydrophobic molecules, such as fatty acids, ethanol, and benzene may enter the cell by becoming dissolved in the lipid phase of the membrane. Conversely, most water-soluble molecules, such as glucose, nucleosides, amino acids, and inorganic salts, do not readily cross the cytoplasmic membrane, but instead must be specifically translocated. It is the selective nature of the cytoplasmic membrane and its transport processes that make this structure extremely important to proper cellular function. Solute movement across a membrane can be classified into several categories depending on the mechanism involved and whether the process is active; i.e. requires expenditure of energy.

Passive Transport Across the Cell Membrane

In simple diffusion, a small molecule in aqueous solution dissolves into the phospholipid bilayer, crosses it, and then dissolves into the aqueous solution on the opposite side. The diffusion rate of the molecule across the bilayer is proportional to the concentration gradient across it and to the hydrophobicity of the compound. No specific membrane proteins are involved in the translocation process and this energy-independent passive process results in equilibration of a solute across the membrane. (Darnell *et al.*, 1990).

In facilitated diffusion, membrane proteins are involved in the

translocation of solutes through the membrane; however, this process does not require energy. These proteins span the membrane, with some regions of the protein exposed to the cytoplasm and other regions to the external environment. It is thought that the binding of the solute to a site on the exterior surface of the protein induces a conformational change that generates a gate and, in this way, the solute is released inside the cell (Darnell *et al.*, 1990).

Active Transport

Active transport involves the expenditure of energy and enables a microbial cell to accumulate solutes (without any chemical modification) to high internal concentrations. Substances transported by active transport include some sugars, most amino acids and organic acids, and some inorganic ions such as sulfate, phosphate, and potassium. The energy for driving this process comes from the separation of protons across the membrane (proton-motive force) or from the hydrolysis of ATP (Fig. 1).

The Chemiosmotic Concept

The chemiosmotic theory offers an explanation for the mechanism of coupling energy-generating and energy-consuming processes in the cytoplasmic membrane (Konings *et al.*, 1981). Energy transducing systems like electron transfer systems, ATPase complexes, and solute transport systems act as electrogenic proton pumps. In the cytoplasmic membrane of bacteria, these

proton pumps translocate protons from the cytoplasmic side of the membrane to the external medium. The cytoplasmic membrane is impermeable to ions, especially to H^+ and OH^- . The net result is the separation of electric charges within and across the membrane and the generation of a pH gradient (ΔpH) and an electrical potential ($\Delta \Psi$) across the membrane. The cytoplasm side of the membrane is electrically negative and alkaline, and the outside of the membrane is electrically positive and acidic (Fig. 1). The ΔpH and $\Delta \Psi$ cause the membrane to be energized, and this energy can be converted into work. The energized state of the membrane is normally expressed as the proton-motive force (Δp) and defined by the equation $\Delta p = \Delta \Psi - Z\Delta pH$ (mV), in which Z equals $2.3 RT/F$ (R is the gas constant, T the absolute temperature, and F is the Faraday constant) (Konings *et al.*, 1981).

The chemiosmotic theory considers the energy-transducing systems in the membrane as primary transport systems which convert chemical or light energy into electro-chemical energy. Secondary transport systems are those in which no chemical transformation takes place and only spatial rearrangements change as reactants distribute across the membrane. The theory also postulates that the Δp drives energy-consuming processes in the membrane by a reversed flow of protons. The energy of the Δp is either converted into ATP by reverse action of the ATPase complex in a process

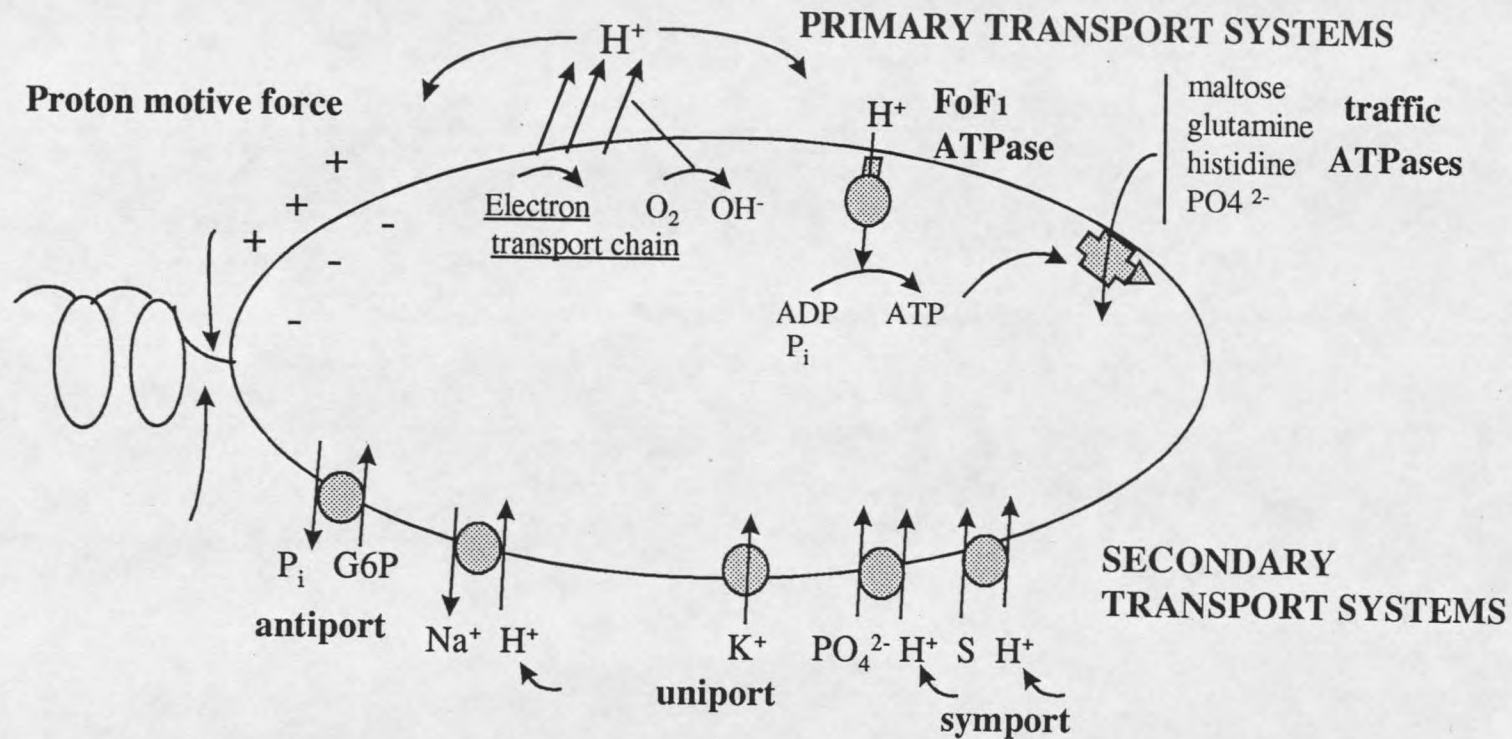


Figure 1. Solute transport in bacteria. The diagram illustrates generation of a proton-motive force (Δp) by H^+ extrusion through the respiratory chain and use of Δp by several paths that perform work: FoF1-ATPase, the flagellar motor, and several kinds of secondary transport systems (proton-linked transport systems). Also shown are examples of primary transport systems that couple movement of substrates to hydrolysis of ATP (traffic-ATPases). Modified from Harold and Maloney (1996).

called oxidative (electron transport) phosphorylation, drives osmotic work such as the formation of solute gradients by secondary transport systems, or facilitates mechanical work such as rotation of a flagellum.

Ionophores

Ionophores are important tools in the study of chemiosmotic systems. They form organosoluble complexes with cations and mediate the transport of the cations across membranes. They are grouped as mobile carriers or as channel formers, according to their mechanism of action. In the case of the mobile carrier mechanism, the orientation of the transport system is altered by solute-binding, either by rotating across the plane of the membrane or serving as a shuttle between outer and inner surfaces. The channel model assumes the carrier protein remains essentially stationary in the membrane and forms a hydrophilic channel or pore specific for a given solute (Reed, 1979).

Ionophores are very useful in the study of chemiosmotic systems since they specifically increase the rate of one or more ion transfer processes across the membrane. For example, individual manipulation of the two components of the Δp can be effected with two potassium ionophores. Valinomycin increases the electrogenic permeability of a membrane for potassium and leads to the dissipation of $\Delta\Psi$ if a high concentration of potassium is present (more than 10 mM). Nigericin catalyzes an

electroneutral potassium proton exchange, dissipating ΔpH under the same conditions (high external concentration of K^+).

Several ionophores are able to prevent utilization of chemical energy derived from respiratory electron transport for net phosphorylation of ADP to ATP (Dawes, 1986). These compounds are called uncouplers and are essentially weak acids. Where a proton gradient exists (inside negative), the uncoupler will become protonated outside the cell and then cross the membrane to the interior where it will be deprotonated because of the alkaline environment. The deprotonated acid will return to the exterior, completing the cycle, thus dissipating the electrochemical gradient by functioning as a ionophore for protons. Examples of uncouplers include 2,4-dinitrophenol (DNP), and carbonyl cyanide m-chlorophenyl hydrazone (CCCP).

Uniport, Symport, and Antiport

Three types of secondary transport are recognized in bacteria (Maloney, 1987) (Fig. 1). When solute transport is mediated by a carrier protein and the substrate movement is independent of any coupling ion, transport is designated as uniport. Symport (or cotransport) occurs when two (or more) different molecules or ions are simultaneously transported by the same carrier in the same direction. In this type of transport, the electrochemical gradient of one solute (usually proton or sodium ion) is used to drive the

uphill transport of the other solute. Antiport (countertransport) is similar to symport, except that the solutes are transported in opposite directions. In the case of symport and antiport, the uptake may be electroneutral or electrogenic depending upon whether the proton translocation is accompanied by the movement of a compensating anion, or by a neutral solute.

An example of uniport is represented by the glycerol facilitator (GlpF) of *E. coli* (Poolman and Konings, 1993). Glycerol uptake is immediately followed by its conversion to *sn*-glycerol 3-phosphate. This allows downhill entry of glycerol that is sufficiently fast for an efficient metabolism. Since glycerol is highly membrane permeable, failure to convert it to *sn*-glycerol 3-phosphate could lead to passive outward diffusion of glycerol down its concentration gradient, creating an useless cycle.

Symport reactions are designed for the accumulation of solutes from the environment, and this class satisfies the demand of many amino acid- and carbohydrate-transport systems. The lactose transport system is the best studied example of symport in bacteria, but several other proton-linked solute symport systems have been described. Among these are the arabinose, galactose, glycine, histidine, pyruvate, succinate, and phosphate porters (Maloney, 1987).

Finally, antiport systems are well adapted for the excretion of undesired solutes (metabolic by-products) from the cytoplasm since solute efflux is directly linked to proton (or sodium) influx. Proton antiporters in bacteria

include the excretion systems for Na^+ , K^+ , and Ca^{2+} ions (Sorensen and Rosen, 1982), and other antiporters catalyze uptake of a solute in a coupled exchange with another solute. The exchanged solutes can be anions such as sugar-phosphate/ phosphate, malate/ lactate, or cations such as arginine /ornithine, or neutral solutes such as lactose/ galactose (Poolman and Konings, 1993). Among bacterial anion-exchange systems, the phosphate-linked examples are the best characterized (Maloney *et al.*, 1990). Each of these accepts an organic phosphate as its primary (high affinity) substrate, but will also accept phosphate, usually with a relatively low affinity. These phosphate-linked anion exchangers are designated GlpT, UhpT, and PgtP and transport glycerol-3-phosphate, hexose-6-phosphates, and phosphoenolpyruvate, respectively.

ATP-coupled Solute Transport Systems

H^+ -translocating ATPase

The ATPase complex is a primary proton pump present in the cytoplasmic membrane of bacteria (Dawes, 1986). This complex can convert the free energy of the Δp into ATP synthesis in a coupled process in which protons are translocated from the external medium into the cytoplasm (Fig. 1). Alternatively, it catalyzes the reversed process in which ATP is hydrolyzed and protons are extruded from the cytoplasm into the external medium. It is composed of two polypeptides designated F_0 and F_1 . F_0 is hydrophobic, composed of three polypeptides, is an intrinsic part of the membrane, and

functions as a proton-conducting channel. This proton channel can be blocked by specific inhibitors like N, N'-dicyclohexyl-carbodi-imide (DCCD) thus preventing ATP synthesis whether mediated by respiration, light or an artificially imposed Δp . DCCD also inhibits the generation of Δp via ATP hydrolysis. The F_1 complex is hydrophilic, comprised of five different polypeptides situated on the internal side of the membrane, and contains the catalytic site for the ATPase.

With only a few exceptions, F_0F_1 is found in the membranes of all bacteria. In aerobes and anaerobes (using nitrate, sulfate, carbonate, etc., as electron acceptors), the enzyme functions to synthesize ATP as part of electron transport phosphorylation. In contrast, in anaerobic cells provided with a fermentable substrate, ATP is produced by substrate-level phosphorylation (ATP is synthesized during specific enzymatic steps in the degradation of an organic compound). The ATPase then operates in the hydrolytic direction, extruding protons and generating a proton potential across the membrane.

P-type ATPases

In addition to the F_0F_1 -ATPases, P-type (formerly called E_1E_2) ATPases have been described (Harold and Maloney, 1996). P-type ATPases hydrolyze ATP to move one or more cation inward or outward or in exchange (H^+ , Na^+ , K^+ , Ca^{2+} , Mg^{2+} , or Cu^{2+} are known substrates). The catalytic subunit of P-type

ATPases is phosphorylated by ATP at a conserved aspartyl residue, forming an acylphosphate intermediate during the catalytic cycle. Ion transport therefore involves cyclic transformation of the enzyme between phosphorylated and dephosphorylated species driven by formation and destruction of the high-energy acylphosphate bond.

Transport Systems Utilizing Periplasmic Binding Proteins

Binding protein-dependent transport systems belong to a large superfamily of carriers present in both prokaryotes and eukaryotes (Tam and Saier, 1993). They are otherwise referred to as ABC transporters, and are primary pumps that couple hydrolysis of ATP to movement of substrates across the membrane (Fig. 1). They are also called ABC transporters based on the presence of a conserved ATP-Binding Cassette, a structural stretch of high homology which is invariably found in these proteins (Boos and Lucht, 1996).

These carriers are widespread in Gram-negative bacteria, with more than 20 binding protein-dependent systems having been studied in *E. coli* (Boos and Lucht, 1996); their occurrence in Gram-positive has been reported as well (Tam and Saier, 1993). Binding protein-dependent systems of Gram-negative bacteria are typically a complex of two to four membrane-bound proteins associated with a soluble periplasmic protein (Boos and Lucht, 1996). They typically transport with high affinity, achieving very large concentration gradients. Treatments such as osmotic shock release binding proteins, along with other periplasmic proteins, into the external medium. Loss of the

appropriate binding protein results in a sharp reduction in transport rate or a complete inhibition of transport activity (Rosen, 1987).

The first step in the transport process involves the initial binding of the substrate to the periplasmic-binding protein (Prossnitz *et al.*, 1989). Because of the high concentration of binding protein in the periplasm and because of its high binding affinity, the solute is presented in a concentrated form to the membrane-bound complex. Two members of the membrane-associated complex are hydrophobic proteins that span the membrane and are thought to form a transport channel. The third membrane-bound peptide is a hydrophilic protein that contains an ATP-binding motif (Higgins *et al.*, 1985).

The most accepted model that explains the translocation process in this class of transporters suggests the binding protein interacts with the membrane complex, making direct contact at least with one of the hydrophobic proteins. Interaction of the binding protein at the periplasmic side is by conformational coupling, and transmits a signal to the nucleotide binding domain, thus controlling ATP hydrolysis. ATP hydrolysis results in the induction of conformational changes in the membrane domains which lead to solute translocation (Kramer, 1994). This scenario has not yet been proven. However, in spite of this uncertainty, it is clear the membrane components are able to carry out the translocation step, and the catalytic activity of the cytoplasmic nucleotide-binding domain is controlled by the interaction of the periplasmic binding protein component with the membrane domains

(Kramer, 1994).

There has been controversy concerning the mechanism(s) by which energy is coupled to binding protein-dependent transport systems. Berger (1973), and Berger and Heppel (1974) postulated that ATP, or some form of phosphate bond energy, was the driving force for these permeases, whereas the Δp was the driving force for the shock-resistant systems. In their studies, arsenate (which inhibits ATP synthesis) specifically inhibited shock-sensitive systems and those systems were relatively insensitive to uncouplers of oxidative phosphorylation. Also, ATPase mutants could not use substrates such as D-lactate (can provide energy primarily via electron transport) to drive shock-sensitive transport. However, the ATPase mutants could use glucose as an energy source for transport (substrate-level phosphorylation in the Embden-Meyerhof-Parnas pathway). The conclusions drawn from these experiments have been questioned since the results were based on indirect evidence. Specifically, the Δp was not monitored by direct assay and was assumed to have been appropriately high or low under the different set of conditions used.

Several groups have shown a requirement for Δp (Plate, 1979; Singh and Bragg, 1977). Plate (1979) showed that under conditions where cell ATP levels are unchanged but the Δp is decreased by the addition of valinomycin plus K^+ , the activity of the shock-sensitive glutamine transport system is decreased. These results indicate ATP is not enough to energize transport and

that Δp is essential for the active transport of glutamine. Singh and Bragg, (1977) studied the effect of inhibitors and uncouplers on the osmotic shock-sensitive transport systems for glutamine and galactose. They postulated that an electrochemical gradient of protons formed by ATP hydrolysis through the ATPase complex drives the uptake of these compounds.

Berger and Heppel's (1974) suggestion that direct hydrolysis of ATP was the driving force of binding protein-dependent transport systems was reinforced when it was demonstrated that a nucleotide-binding site exists on one of the membrane components of osmotic shock-sensitive maltose, histidine, and oligopeptide transport systems (Higgins *et al.*, 1985). Direct interaction of ATP with the transport protein was demonstrated by binding studies with ATP and structural analogs (Higgins *et al.*, 1985).

Further evidence supporting the involvement of ATP as the energy source for shock-sensitive transport systems was provided when the histidine and maltose permeases in whole *E. coli* cells were studied (Joshi *et al.*, 1989). In these experiments, transport was unaffected after dissipation of the Δp , while a high ATP pool was maintained, implying that the Δp was unnecessary. Starvation experiments indicated that both transport systems require ATP and that a normal level of Δp was not sufficient. Inhibition of ATP synthesis by arsenate eliminated transport through both permeases, confirming the need for ATP. It was also demonstrated that the simultaneous reduction of the Δp and inhibition of histidine transport by

valinomycin plus K^+ (Plate, 1979) were artifactual, since this effect was observed with histidine permease but not with the maltose permease. These results suggest that some of the shock sensitive transport systems (namely, glutamine and histidine) might be sensitive to a secondary effect of valinomycin.

The development of proteoliposomes (transport proteins are incorporated into pre-formed lipid vesicles) reconstituted from purified binding protein-dependent permeases gave definitive evidence of ATP hydrolysis during active transport (Bishop *et al.*, 1989). Using this system, it was shown that internally trapped ATP allowed active uptake upon addition of binding protein. Proteoliposomes did not catalyze significant ATP hydrolysis until histidine transport was initiated by addition of substrate along with the histidine binding protein. The reconstituted system was completely dependent on all four permease proteins and on internal ATP. Dissipators of the membrane potential had no effect on transport. The demonstration that ATP was hydrolyzed only concomitantly with substrate transport confirmed that ATP drives solute transport in these permeases.

Phosphate Transport Systems

E.coli has two major P_i transport systems: the Pit (phosphate inorganic transport), and the Pst (phosphate specific transport) (Willsky and Malamy, 1980a).

Pit System

When P_i is in excess, P_i is taken up by the low-affinity Pit system. The Pit transporter belongs to the group of permeases that are energized by Δp (Willsky and Malamy, 1980a) and is thought to consist of a single transmembrane protein. All known *pit* mutations map to the same locus in the *E. coli* chromosome and are complemented by transformation with a plasmid carrying a small fragment of DNA which directs the synthesis of a single protein (Elvin *et al.*, 1987). Expression of *pit* is not affected by the P_i concentration in the medium and therefore is expressed constitutively. The Pit transporter in *E. coli* has a K_m of 38 μM and a V_{max} of 55 $nmol P_i \cdot min^{-1} \cdot mg$ cell dry weight⁻¹ and is sensitive to arsenate (Willsky and Malamy, 1980a). It does not require a binding protein and therefore is fully active in spheroplasts (Rosenberg *et al.*, 1977) and in osmotically shocked cells. Since P_i transport through the Pit system is coupled exclusively to the Δp , it is completely abolished by ionophores and uncouplers of respiration (Rosenberg *et al.*, 1977). Willsky and Malamy (1980b) reported that strains of *E. coli* containing the Pit system ceased growth in the presence of arsenate because they accumulated this ion. Arsenate is a toxic analog of P_i in many intracellular reactions and thus inhibits metabolism and growth. Bennett and Malamy (1970) isolated *E. coli* mutants resistant to toxic concentrations of arsenate. These mutants lost the capacity to transport P_i and arsenate by the Pit system.

The low-affinity P_i transport system of *Acinetobacter johnsonii* has been well characterized and it operates in a similar fashion as the Pit system in *E. coli* (Van Veen *et al.*, 1993). A metal phosphate is transported in symport with H^+ and is dependent on the presence of divalent cations, such as Mg^{2+} , Ca^{2+} , Co^{2+} , or Mn^{2+} which form a soluble, neutral metal phosphate ($MeHPO_4$) complex (Van Veen *et al.*, 1994). The Pit transport system in both *E. coli* and *A. johnsonii* is reversible and therefore allows an exchange of P_i inside and outside the cell. It mediates efflux and homologous exchange of metal phosphate, but not heterologous metal phosphate exchange with P_i , glycerol-3P, or glucose-6P. The absence of the heterologous exchange reactions is consistent with the specificity of Pit for metal phosphate. Because this system facilitates reversible movement of P_i across the cytoplasmic membrane, it provides these microorganisms with a major route for the entry and exit of divalent metal ions and P_i .

Pst System

The Pst system has a K_m of $0.43 \mu M$ and a V_{max} of $16 \text{ nmol } P_i \cdot \text{min}^{-1} \cdot \text{mg cell dry weight}^{-1}$ and is a traffic ATPase transporter (Willsky and Malamy, 1980b). The high affinity of this permease for P_i is also reflected by its low rate of arsenate transport; Pst^- strains are able to grow in arsenate-containing medium, but at a reduced growth rate (Willsky and Malamy, 1980b). The *pst* genes are co-regulated as members of the PHO regulon and are induced more

than 100-fold during P_i limitation (Wanner, 1993). PstS is the periplasmic P_i -binding protein. It binds P_i and transfers it to the Pst permease complex, which is a transmembrane structure consisting of two proteins, PstA and PstC in association with the permease PstB. The deduced amino acid sequence of PstB contains the consensus sequence for an ATP binding domain (Torriani, 1990), and therefore it is believed the Pst system is energized by ATP or a related nucleotide.

Organophosphate Uptake

Three uptake systems for organophosphates are present in *E. coli*: Ugp and GlpT for glycerol 3-phosphate (G3P) and UhpT for hexoses 6-phosphates (Wanner, 1996). Each of these accepts their respective organic phosphate, and each also accepts P_i , but at 10-fold reduced affinity relative to the organic substrate (Maloney *et al.*, 1990).

Synthesis of the Ugp system is inducible by P_i limitation, and is under PHO regulon control (Brzoska *et al.*, 1987). Genes *ugpB*, *ugpA*, *ugpE* and *ugpC* form an operon. *ugpB* codes for the periplasmic binding protein for G3P, *ugpA* and *ugpE* code for the two membrane-bound components, and *ugpC* codes for a protein containing the ATP-binding domain and is thought to be the energy module of the system. For maximal expression of *ugp*, the cells have to be induced by P_i starvation or mutants have to be used that are expressing the PHO regulon constitutively (*phoR* or *pst*). The observation that mutants in *phoB* as well as double mutants in *phoR* and *CreC* prevent

expression, identified the *ugp* transport system as a member of the PHO regulon.

GlpT and UhpT belong in the category of P_i -linked antiport. Each of these is a single-component system that transports its respective organophosphate substrate in an unaltered form (Maloney *et al.*, 1990). Each system mediates the self-exchange of P_i ($P_i:P_i$ exchange) and each system catalyzes the heterologous exchange of P_i and some sugar phosphate (P_i : G6P, P_i : G3P). Synthesis of these antiporters is inducible by a substrate specific for that transporter. Therefore since P_i is not a specific substrate, none of these anion exchangers is considered to play a role in P_i uptake if the specific inducer is not present.

Summary of Transport of Phosphate and Phosphorylated Compounds in *Escherichia coli*

There are two systems for the active transport of P_i , two for glycerol 3-phosphate (G3P), and one for hexoses 6-phosphates (Table 1). Among these systems, it is appropriate to distinguish between those which operate when there is high P_i in the medium and those which operate when the cells are growing under P_i limiting conditions. When P_i is abundant, the Pit system operates as the major active transport system for P_i , GlpT transports G3P, and UhpT transports hexose phosphates. These systems are relatively simple and their affinities for their substrates is low - in the range of 20 to 100 μ M.

When the concentration of P_i in the medium drops below 5 μ M, the

PHO regulon is induced. Along with AP and several other components of the PHO regulon, two additional, more efficient, but more complex transport systems are turned on: Pst, which is specific for P_i , and Ugp, which is specific for G3P. The affinities of these transporters for these substrates are very high as it would be expected when scavenging scarce sources of phosphate is required.

Table 1. Transport systems of inorganic phosphorus, Glycerol-3-Phosphate, and hexoses phosphates in *Escherichia coli*

Condi- tions	Substrate	Transport system	membrane transport genes	Affinity constant (μ M)	Regu- lation
High P_i	P_i	Pit	<i>pit</i>	38	no
	G3P	GlpT	<i>glpT</i>	12	yes
	Hexose	UhpT	<i>uhpT</i>	100	yes
	Phosphate				
Low P_i	P_i	Pst	<i>pstS, pstC, pstB, pstA</i>	0.43	yes
	G3P	Ugp	<i>ugpD, ugpB, ugpA, ugpC</i>	2	yes

Modified from Yagil (1987).

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CHAPTER 3

Rhizobium tropici HAS TWO PHOSPHATE-REPRESSIBLE PHOSPHATE
TRANSPORT SYSTEMSIntroduction

Nitrogen fixation in legume nodules involves a complex exchange of nutrients between the plant and the bacteroid. This exchange involves transport across the bacteroid membrane and the plant-derived envelope surrounding the bacteroid, the peribacteroid membrane. Information on the transport of compounds across these membranes is valuable because it will aid in our understanding of how the bacteroids retain a mutualistic relationship with the host plant. 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 remains to be well characterized. Given the significant metabolic activity of the bacteroids, phosphorus supply to the bacteroids may be critical for optimum symbiotic function, and understanding mechanisms by which bacteroids take up phosphorus will provide useful information regarding phosphorus exchange

between the symbionts and phosphorus flow within the symbiosis.

The transport of inorganic phosphorus (P_i) has been investigated in various bacteria. Two P_i transport systems have been characterized in *Escherichia coli* (Rosenberg *et al.*, 1977; Willsky and Malamy, 1980), *Acinetobacter johnsonii* (Van Veen *et al.*, 1993), and *Pseudomonas aeruginosa* (Lacoste *et al.*, 1981). In each case, a constitutively expressed low-affinity transport system and a P_i -repressible high-affinity permease have been identified. The low-affinity P_i transport system is energized by proton motive force and consists of a single membrane component (Elvin *et al.*, 1987). In contrast, the high-affinity P_i transporter is a multicomponent system consisting of several membrane proteins and a periplasmic binding protein (reviewed by Wanner, 1996).

In other microorganisms only a single system has been described. This is the case for *Micrococcus lysodeikticus* (Friedberg, 1977), as well as for several species of *Rhizobium* (Smart *et al.*, 1984; Bardin *et al.*, 1996). In these organisms, synthesis of the P_i transport system is also induced by P_i limitation, but the K_m values are typically an order of magnitude larger than that reported for the inducible high affinity systems in bacteria that contain two P_i uptake mechanisms.

A preliminary description of phosphorus metabolism in *R. tropici* strain CIAT899 has been reported (Al-Niemi *et al.*, 1997). As with other Gram-negative bacteria (Wanner, 1996), this organism exhibits a significant

increase in P_i transport rate and phosphatase activity in response to P_i limitation; this induction occurs when the medium P_i concentration decreases below 1 μM (Al-Niemi *et al.*, 1997). In this report, transport of P_i in *R. tropici* is described. In contrast to other rhizobia previously studied (Smart *et al.*, 1984; Bardin *et al.*, 1996), this strain of rhizobia has two distinct functional P_i transport systems. In addition, *R. tropici* appears novel relative to all other bacteria thus far investigated in that both inorganic phosphorus transport systems are inducible by P_i stress, are shock sensitive, and are energized by phosphate bond energy.

Materials and Methods

Strains and Culture Conditions

Strains CIAT899 and CAP45 were used in all experiments. CIAT899 is the type strain for *R. tropici* type IIB (Martinez-Romero *et al.*, 1991), and CAP45 is a P_i transport mutant derived from CIAT899 (see below).

CIAT899 was maintained on minimal mannitol ammonium agar medium (MMNH_4) containing 55 mM Mannitol, 9.3 mM NH_4Cl , 10 mM KH_2PO_4 , 1.8 mM Na_2SO_4 , 1.8 mM K_2SO_4 , 37 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.68 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 8.2 μM Biotin, 5.9 μM thiamine, 10 mM MOPS [3-(N-morpholino) propane-sulfonic acid] and 5 mM MES [2-(4-morpholino)-ethane-sulfonic acid)], final pH 7.2. CAP45 was maintained on the same

medium, except β -glycerol-phosphate (β GP) replaced mannitol as the sole carbon source, and gentamicin was included at a final concentration of $25 \text{ mg} \cdot \text{liter}^{-1}$.

The P_i transport mutant CAP45 was isolated using methodology similar to that reported by Torriani and Rothman (1961) for the isolation of PHO regulatory and P_i transport mutants of *E. coli* (McDermott, unpublished data). Briefly, *R. tropici* CIAT899 was mutated with transposon Tn5-B22 (Simon *et al.*, 1989) as previously described (De Bruijn and Lupski, 1984). Mating mixtures were plated onto minimal- β GP- NH_4 agar containing gentamicin ($25 \text{ mg} \cdot \text{liter}^{-1}$). To be used as a carbon source, β GP must first be dephosphorylated at rates sufficient to supply glycerol for growth. One candidate phosphatase in CIAT899 would be alkaline phosphatase. However, because of the high concentration of P_i in this medium, growth requires synthesis of alkaline phosphatase under conditions where it is normally repressed. Pure culture isolates of putative regulatory mutants were obtained by subculture onto minimal- β GP- NH_4 -gentamicin agar, and then assayed for P_i transport to determine which regulatory mutants also were defective in P_i transport.

Transport Assays

Early stationary phase cultures were washed twice in MMNH_4 containing no phosphate (pH 7.2) ($\text{MMNH}_4\text{-OP}$), and resuspended in $\text{MMNH}_4\text{-OP}$ to an optical density of $A_{595} = 0.60$. To obtain P_i -starved cells ($-\text{P}_i$ cells), washed cells were incubated in $\text{MMNH}_4\text{-OP}$ at 30°C for 7 hours to allow for maximum induction of P_i transport (Al-Niemi *et al.*, 1997). Chloramphenicol was then added ($50 \mu\text{g} \cdot \text{ml}^{-1}$) to stop further protein synthesis. Nonstarved cells ($+\text{P}_i$ cells) were prepared the same way except chloramphenicol was added immediately after washing and the cells were used within one hour. In similar experiments, it was found that chloramphenicol inhibits induction of alkaline phosphatase for at least 5 h, and thus it was concluded that *de novo* protein synthesis in $+\text{P}_i$ cells did not occur.

The transport assay was conducted in an orbital shaker water bath at 30°C . Washed cells were diluted in $\text{MMNH}_4\text{-OP}$ to $0.125 \text{ mg cell dry weight} \cdot \text{ml}^{-1}$ for $+\text{P}_i$ cells or $0.025 \text{ mg cell dry weight} \cdot \text{ml}^{-1}$ for $-\text{P}_i$ cells. Cell concentration for the $+\text{P}_i$ -treated cells was increased five-fold to ensure sensitive and accurate uptake measurement. After 5 min preincubation, the transport assay was initiated by adding P_i (at concentrations specified in the figure legends) as $[^{32}\text{P}]\text{KH}_2\text{PO}_4$ (specific activity of $22.5 \mu\text{Ci} \cdot \mu\text{mol}^{-1}$; filtered prior to use to remove any ^{32}P adsorbed to extraneous particles). Cell samples

(0.5 ml) were withdrawn at 20 sec. intervals, collected on a 0.3 μ m glass fiber filter (Gelman Sciences, Ann Arbor, Michigan), and washed with 20 ml of transport rinse buffer (TRB), [20 mM MES, 5 mM KH_2PO_4 (pH 6.5)]. Filters were placed in counting vials, 20 ml of H_2O added, and the radioactivity retained was measured by Cerenkov radiation (Haviland and Bieber, 1970).

Phosphate Exchange and Efflux

P_i -starved cells were loaded for 4 min with [^{32}P] KH_2PO_4 (either 5 μM or 400 μM) at 30°C, and diluted 100-fold into $\text{MMNH}_4\text{-OP}$ without (efflux experiments) or with either 25 μM or 2 mM unlabeled potassium phosphate (exchange experiments). At given time intervals, 0.5 ml samples were filtered and washed as described above.

Osmotic Shock Treatment

An osmotic shock procedure similar to that described by Neu and Heppel (1965) was used. Cells were washed twice with 30 mM Tris (pH 8.0) and resuspended to a cell density of 5 mg cell dry weight $\cdot \text{ml}^{-1}$ in 30 mM Tris (pH 8.0) containing 1 M sucrose and 10 mM EDTA. Following 15 min. incubation at room temperature, cells were collected by centrifugation for 4 min at 14,000 $\times g$, and then periplasmic proteins released by resuspending the pellet in 0.1 mM MgSO_4 at room temperature. The shock-treated cells were collected by centrifugation, resuspended in $\text{MMNH}_4\text{-OP}$ and used for P_i transport assays.

To verify the release of periplasmic enzymes, alkaline phosphatase activity and protein concentration were determined in the supernatant of the shock-treated cells. In addition, the cytoplasm marker enzyme malate dehydrogenase was measured to determine if lysis of cells occurred. The release of periplasmic proteins and extent of cell lysis was quantified with the same assays of cleared extracts of sonicated samples that contained equivalent amounts of shocked cells. Alkaline phosphatase activity was measured by recording the hydrolysis of *p*-nitrophenyl phosphate (ρ NPP) at 415 nm (Smart *et al.*, 1984). Malate dehydrogenase activity was assayed at 340 nm as the rate of NADH oxidation. The 350 μ l reaction contained 1.5 mM oxalacetic acid, 0.25 mM NADH, 10 mM K_2HPO_4 (pH 7.5), and 50 μ l of shock fluids or cell extract. Both assays were conducted in a BioRad 3550-UV microplate reader. Protein concentration was determined using a protein assay kit (Bio-Rad).

EDTA Treatment of Cells

EDTA treatment facilitated the incorporation of inhibitors and the membrane potential indicator, tetraphenyl phosphonium bromide (TPP^+) into the cytoplasmic membrane. $-P_i$ cells were washed twice with 30 mM Tris (pH 8.0), resuspended to a density of 5 mg cell dry weight $\cdot$ ml $^{-1}$ in 30 mM Tris, and EDTA (pH 7.0) was then added to 1 mM. After 5 min incubation at room temperature, cells were centrifuged for 4 min at 14,000 $\times g$, washed twice with 30 mM Tris, and resuspended in $MMNH_4$ -OP at a density of 0.5 mg cell dry

weight $\cdot$ ml⁻¹. Alkaline phosphatase was not released by this procedure (data not shown), indicating the EDTA treatment did not result in release of periplasmic proteins.

Determination of Membrane Potential ($\Delta\Psi$)

Ionophore dissipation of membrane electrical potential ($\Delta\Psi$) (interior negative) was measured as the distribution of radiolabeled TPP⁺ across the membrane (Rottenberg, 1979; Kashket, 1985). TPP⁺ uptake was measured in parallel experiments, with and without inhibitors, using the same medium and conditions described above for P_i uptake, except choline chloride was added at a final concentration of 50 mM. Choline chloride reduces binding of TPP⁺ to anionic groups at the cell surface (Maloney, 1983). Cell concentration in the TPP⁺ uptake assays was increased five-fold to ensure sensitive and accurate uptake measurement. Accordingly, inhibitor concentrations were proportionally increased so assay conditions were the same used in P_i transport measurements. The inhibitor assays consisted of MMNH₄-OP containing the specified inhibitor and 0.125 mg dry weight $\cdot$ ml⁻¹ of EDTA-treated -P_i cells. After 5 or 45 min of incubation at 30°C with constant shaking, [³H] TPP⁺ (27.5 μ Ci $\cdot$ μ mol⁻¹) was added to a final concentration of 18 μ M. At the indicated times, 0.5 ml samples were withdrawn and filtered through a 0.3- μ m glass fiber filter and washed with 20 ml of TRB. Filters were

placed in counting vials, 20 ml of scintillation cocktail added (Scintisafe PlusTM 50%, Fisher Chemical), and radioactivity measured in a TRI-CARB liquid scintillation analyzer (model 4430, Packard Instrument Co.).

ATP Determination

Intracellular ATP concentrations were determined in experiments where P_i and TPP^+ uptake inhibitors were studied. The reaction mixtures were identical to transport assays, except no radioisotope was added. After the indicated incubations, cellular ATP was extracted as described by Joshi *et al.*, (1989). Briefly, 0.5 ml of assay mixture was added to 0.25 ml of ice-cold 24% (v/v) perchloric acid, incubated on ice for 20 min. and then centrifuged at 15,000 $\times g$ for 4 min. A 0.5 ml aliquot of supernatant was transferred to a new microcentrifuge tube and neutralized with 0.125 ml of 4 M KOH and 0.125 ml of 2 M $KHCO_3$. After 30 min on ice, perchlorate was sedimented by centrifugation at 15,000 $\times g$ for 1 min. The supernatant samples were stored at $-20^\circ C$ for 24 h.

After thawing at room temperature, samples were centrifuged at 14,000 $\times g$ for 10 sec. to sediment residual perchlorate. ATP concentration was determined using the luciferase assay, measuring light emission in a Turner TD-20e luminometer, and employing the internal standard technique (Turner Designs, 1983). The ATP assay contained 0.05 ml perchloric acid-treated supernatant, 0.1 ml 10 mM Tris buffer (pH 8.0), and 0.1 ml luciferase-

luciferin (Promega). After luciferase was injected into the sample, and a light measurement taken, an ATP standard was added to the same cuvette and a second light measurement taken. The amount of ATP in the sample is calculated using the following equation:

$$\text{ATP (pmol /50 } \mu\text{l)} = \frac{\text{RU} - \text{RB}}{\text{RIS} - \text{RU}} \times \text{ATP concentration (pmol /50 } \mu\text{l) in standard}$$

where RU represents the luminescence reading for the sample, RB is the luminescence reading of the blank, and RIS is the luminescence reading after the addition of the internal standard.

Results

Isolation of the Phosphate Transport Mutant CAP45

Tn5B22 mutagenesis of *R. tropici* and selection on minimal β GP-gentamicin agar resulted in a collection of 43 gentamicin-resistant mutants that were found to be constitutive for the expression of alkaline phosphatase (CAP) (T.R. McDermott, personal communication). Presumably, constitutive expression of alkaline phosphatase was due to lack of normal repressive regulatory mechanisms. Screening these mutants for a P_i transport phenotype identified several mutants that were defective in up-regulation of P_i transport in response to P_i starvation. Southern blot analysis of chromosomal DNA prepared from the P_i transport mutants verified the presence of Tn5B22 (T.R. McDermott, personal communication). In *E.coli*,

phoU (some exceptions), *pstA* and *pstS* mutants express alkaline phosphatase constitutively; however, only *pstA* mutants are impaired in up-regulation of P_i transport (Torriani, 1990; Wanner, 1993). Based on these observations with *E. coli* and subsequent experiments (described below), the isolate selected for further characterization and comparison to the wild-type strain was tentatively designated as a *pst* mutant and is referred to as CAP45.

Kinetic Parameters of Phosphate Uptake

As measured in the range of 0.1 to 500 $\mu\text{M } P_i$, linear regression analysis of initial P_i transport velocities in $-P_i$ cells revealed the presence of two P_i transport systems in CIAT899 (Fig. 2). Kinetic studies revealed the K_m values of these two transport systems differed by approximately two orders of magnitude. Both systems were expressed under both $+P_i$ and $-P_i$ growth conditions, and both systems were induced in response to P_i deprivation (Table 2).

Only a single transport system was evident in CAP45 (Fig. 2, Table 2), providing an opportunity to study it in the absence of the other system that would otherwise influence overall P_i transport behavior. The K_m for this P_i permease was 34 μM , suggesting it is the low-affinity system. The V_{max} for this low-affinity system present in CAP45 was similar to that found with CIAT899 under both $+P_i$ and $-P_i$ growth conditions, although V_{max} estimates for CAP45 suggested the increase in P_i transport by the low-affinity system in response to P_i stress was more substantial than was apparent in the wild-type

strain CIAT899. Based on these K_m estimates, concentrations of 5 μM and 400 μM P_i were used in subsequent experiments to evaluate P_i uptake by the high-affinity and low-affinity P_i transport systems, respectively, when determining the influence of various treatments.

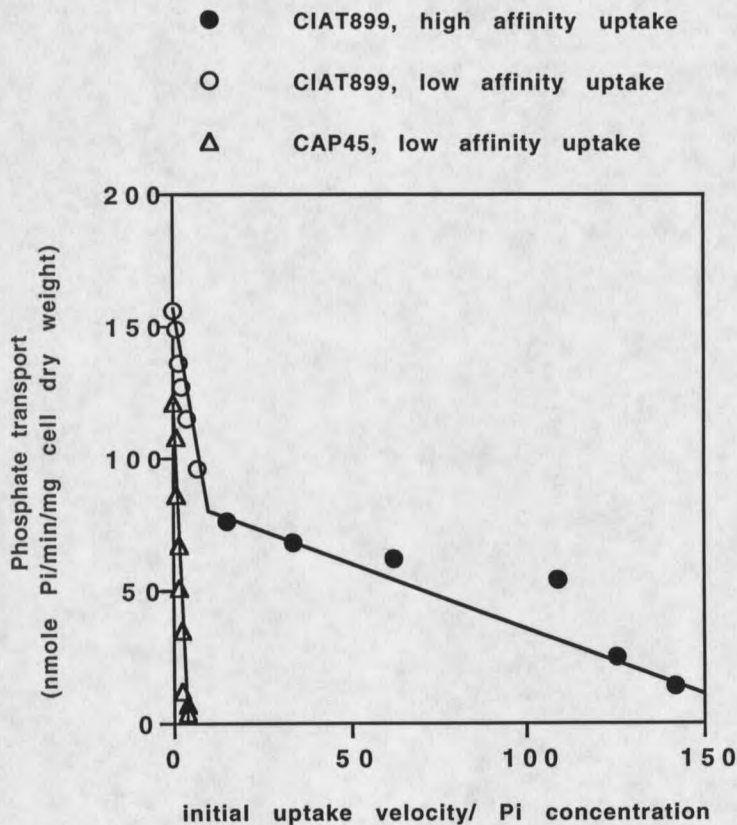


Figure 2. Eadie-Hofstee plots of initial P_i uptake velocities in *R. tropici* CIAT899 and CAP45. Transport rates were determined with $-\text{P}_i$ cells as described in Materials and Methods at P_i concentrations between 0.1 and 500 μM . Each point represents the mean of three independent experiments, each having three replicates. The standard errors for each data point did not exceed 10% of their respective mean.

Table 2. Kinetic parameters of P_i uptake in *R. tropici* CIAT899 and CAP45. The kinetics of P_i uptake was analyzed using Eadie-Hofstee plots. Initial velocities in $+P_i$ and $-P_i$ cells were determined over the first 20 and 10 sec., respectively. Values are the mean $\pm$ standard error of three separate experiments, each having three replicates.

Strain	Growth	High-affinity uptake		Low-affinity uptake	
		Km (μ M P_i)	Vmax (nmol of P_i /min/mg cell dry wt)	Km (μ M P_i)	Vmax (nmol of P_i /min/mg cell dry wt)
CIAT899	$-P_i$	0.45 $\pm$ 0.01	86.2 $\pm$ 4.9	9.6 $\pm$ 1.0	153.8 $\pm$ 13.6
	$+P_i$	0.34 $\pm$ 0.02	0.22 $\pm$ 0.01	35.7 $\pm$ 1.0	1.33 $\pm$ 0.01
CAP45	$-P_i$	ND*	ND	34.3 $\pm$ 2.7	118.0 $\pm$ 7.5
	$+P_i$	ND	ND	33.1 $\pm$ 1.3	0.41 $\pm$ 0.02

*ND, not detected

Effect of Osmotic Shock on Phosphate Uptake

In osmotic shock treated cells, P_i uptake was dramatically reduced (Fig. 3). As determined with high and low P_i levels that were saturating for either P_i transport system, osmotic shock inhibited P_i transport rates by approximately 80%. This was the case for both strains and suggested that both P_i transport systems are dependent on one or more P_i -binding proteins. P_i uptake in $+P_i$ cells was unaffected by osmotic shock. Kinetic analysis of osmotically shocked cells confirmed the requirement of a P_i -binding protein for both systems (Table 3). Comparing kinetics estimates of shocked $-P_i$ cells with kinetics estimates of control cells (Table 2) demonstrates that loss of the putative P_i -binding protein(s) does not influence the affinity of either permease for P_i , but velocities are significantly reduced. This comparison was made using the high-affinity P_i transport system of the wild-type CIAT899 and the low-affinity transporter of the mutant CAP45, because in the mutant strain the low-affinity transporter can be studied in the absence of the other system that would influence overall P_i transport activity.

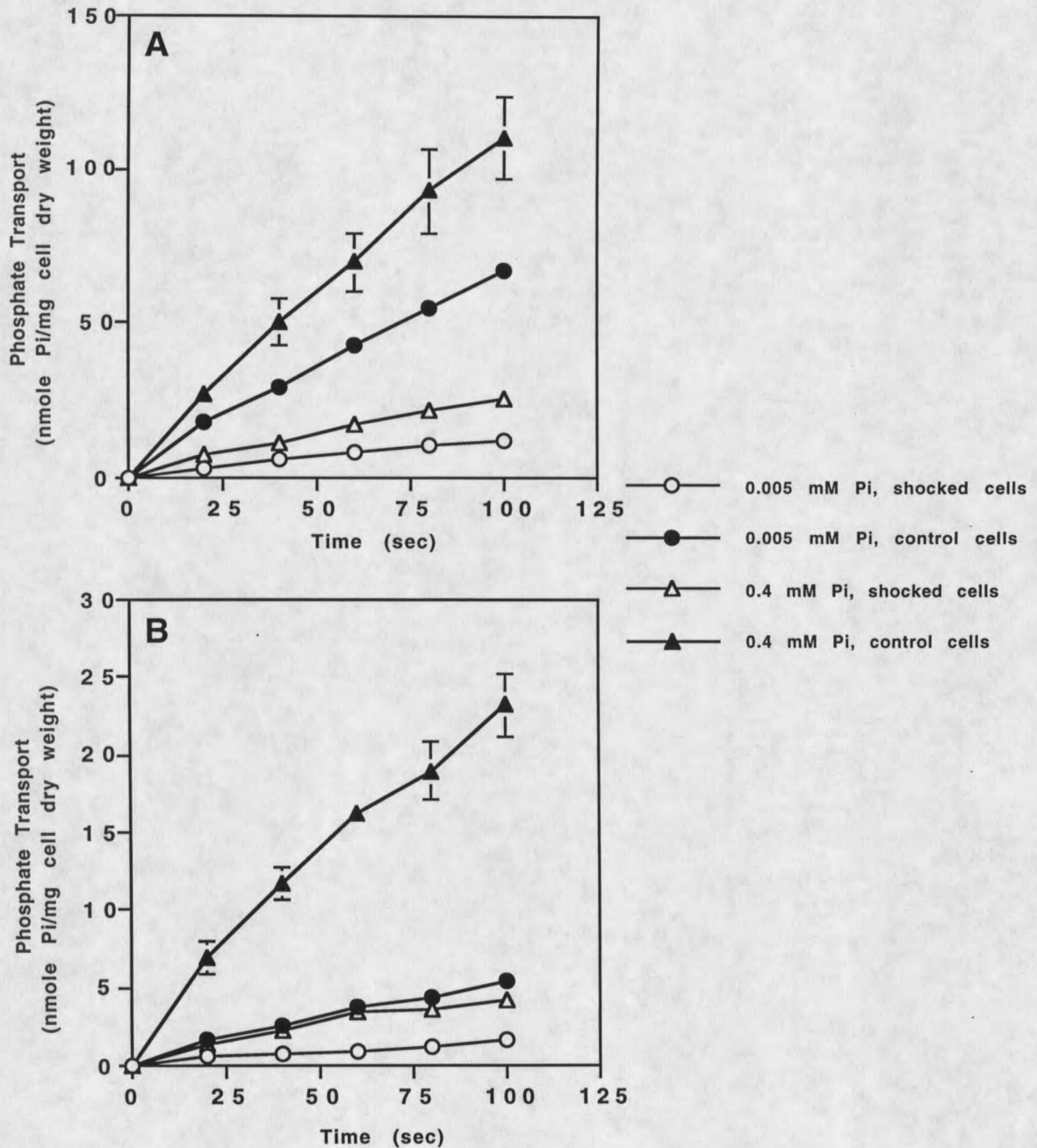


Figure 3. Effect of osmotic shock on the uptake of P_i in $-P_i$ cells of *R. tropici* CIAT899 and CAP45. The uptake of P_i was determined in control cells and shocked cells at concentrations of 5 μM P_i and 400 μM P_i . (A) P_i uptake in the wild-type strain CIAT899; (B) P_i uptake in the mutant CAP45. Each point represents the mean of three replicate experiments (wild type) or two replicate experiments (mutant). Error bars, where visible, represent one standard error.

Table 3. Kinetic parameters of P_i uptake in osmotic shock-treated cells. The kinetics of P_i uptake was analyzed using Eadie-Hofstee plots. Initial velocities in osmotic shocked $-P_i$ cells were determined over the first 10 sec at P_i concentrations between 0.1 and 500 μM . Values are the mean $\pm$ standard error of two separate experiments, each having three replicates.

Strain	Growth	High-affinity uptake		Low-affinity uptake	
		Km ($\mu M P_i$)	Vmax (nmol of P_i /min/mg cell dry wt)	Km ($\mu M P_i$)	Vmax (nmol of P_i /min/mg cell dry wt)
CIAT899	$-P_i$	0.43 $\pm$ 0.05	17.5 $\pm$ 2.5	19.3 $\pm$ 0.6	52.5 $\pm$ 6.7
CAP45	$-P_i$	ND*	ND	37.4 $\pm$ 1.9	2.12 $\pm$ 0.06

* ND, not detected

The release of periplasmic proteins and structural integrity of osmotic shocked cells was verified by assaying for activity of alkaline phosphatase and malate dehydrogenase, respectively (Table 4). The supernatant of pelleted shocked $-P_i$ cells contained 19% of total cellular protein and 16 % of alkaline phosphatase activity; the protein concentration and alkaline phosphatase activity in the same quantity of control cells was less than 1% of shocked cells. The combination of relatively high levels of alkaline phosphatase and proteins in the supernatant of the shock-treated cells was taken as an indication that the cells had 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 the shock treatment did not lyse the cells (Table 4).

Table 4. Release of periplasmic proteins from CIAT899 by osmotic shock treatment. $-P_i$ cells were shocked as described in Materials and Methods. Control cells were treated as shocked cells, except neither EDTA nor sucrose were used. Data are from a single representative experiment.

Treatment		Protein content, and enzyme activities found in the shock or control supernatants		
		Protein concentration (μg protein/ml extract)	Alkaline Phosphatase (nmol/min/ml extract) ^a	Malate Dehydrogenase (nmol/min/ml extract) ^b
Control cells		0.47 (0.3) ^c	14.86 (0.78)	0 (0)
Osmotic shocked		54.5 (19.2)	181.78 (15.6)	0 (0)
Sonicated cells	control	354.3 (99.8)	1880 (99.2)	321 (100)
	osmotic	228.6 (80.7)	982.9 (84.4)	284 (100)

^a nmol of *p*-nitrophenyl phosphate hydrolyzed

^b nmol of NADH oxidized

^c percentage of total cellular protein or enzyme activity is shown in parentheses.

Energy Coupling to Phosphate Transport

The uncoupler carbonyl cyanide-*m*-chlorophenyl hydrozone (CCCP) dissipates the energized membrane state and inhibits processes that use the proton-motive force (Δp) directly as a source of energy. Reactions driven directly by phosphate bond energy should be resistant to the action of this compound. Conversely, *N*, *N'*-dicyclohexyl-carbodi-imide (DCCD) should significantly reduce ATP levels but leave Δp relatively unaffected. On this basis, the effects of reducing the membrane potential and ATP levels on P_i transport were studied.

Under the conditions of the assays (pH 7.2), neutrophilic bacteria such as rhizobia do not generate a significant chemical potential (ΔpH); therefore the Δp consists primarily of the electrical membrane potential ($\Delta\Psi$) alone (Kashket, 1985). The $\Delta\Psi$ (interior negative) was monitored by measuring the uptake of radiolabeled tetraphenyl phosphonium bromide (TPP^+). The distribution across the membrane of this lipophilic cation serves as a good indicator of changes in the Δp . The effect of CCCP on the membrane potential is shown in Figure 4.

To assess the roles of Δp and ATP in energizing P_i transport, CIAT899 and CAP45 were treated with the uncoupler CCCP and the ATPase inhibitor DCCD. Table 5 reveals that both the high and low affinity P_i transport systems are strongly inhibited by DCCD, while in general, they are much less sensitive to the uncoupler CCCP. These results indicate that $\Delta\Psi$ is not involved in energizing P_i transport by either system. Conversely, the correlation between low ATP levels and lack of P_i transport suggest that ATP may be directly involved in energization.

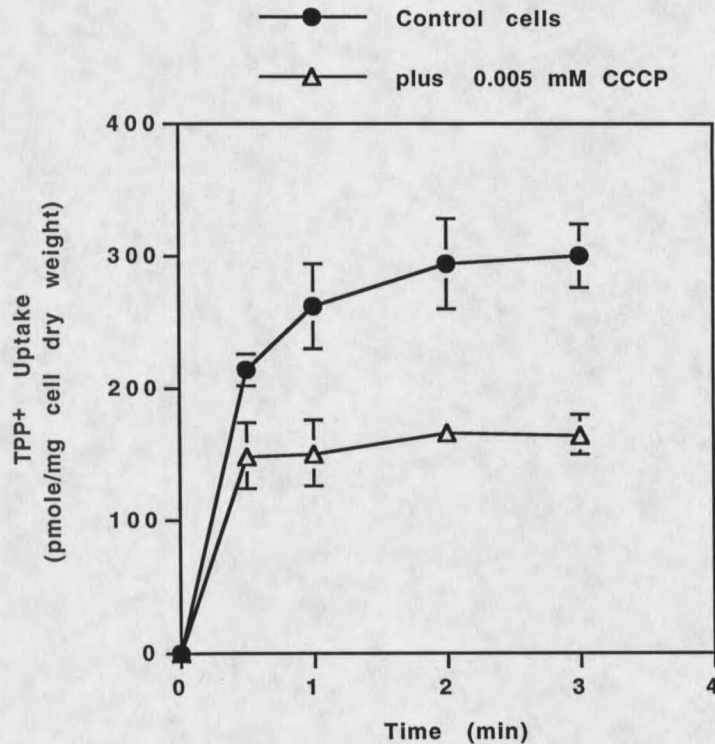


Figure 4. Effect of CCCP on TPP⁺ uptake in *R. tropici* CIAT899. -P_i cells were treated with EDTA as described in Materials and Methods. After 5 min of incubation with the inhibitor CCCP, TPP⁺ uptake was initiated by the addition of [³H]TPP⁺ at a final concentration of 18 μ M (specific activity = 27.5 μ Ci $\cdot$ μ mol⁻¹). Ethanol was used to solubilize the inhibitor and was included in control cells. Cell concentration in this assay was increased five-fold to ensure sensitive and accurate uptake measurement. Accordingly, inhibitor concentrations were proportionally increased so assay conditions were the same used in P_i transport measurements. Each point represents the mean of three replicates. Error bars, where visible, represent one standard error.

Table 5. Effect of Proton Motive Force dissipation and ATP depletion on P_i transport in *R. tropici* CIAT899 and CAP45. $-P_i$ cells were treated with EDTA as described in Materials and Methods, preincubated with the inhibitor and then assayed for P_i uptake and intracellular ATP. Values are means $\pm$ standard error of two separate experiments. Cells were preincubated for 5 min in the presence of the proton dissipator CCCP (A) or 45 min in the presence of the ATPase inhibitor DCCD (B).

Additions	Strain	P _i uptake nmol P _i /min/mg cell dry weight		ATP levels nmol ATP/mg cell dry weight
		5 μM P _i	400 μM P _i	
A				
Ethanol ^a control 5 min	CIAT899	26.7 ± 3.2	41.8 ± 3.1	2.2 ± 0.2
	CAP45	2.39 ± 0.27	8.76 ± 0.08	1.41 ± 0.31
1 μM CCCP	CIAT899	23.8 ± 1.4 (11) ^b	44.9 ± 2.1 (0)	1.84 ± 0.08 (17.8)
	CAP45	2.11 ± 0.06 (11.7)	12.2 ± 0.1 (0)	1.35 ± 0.26 (4.2)
B				
Ethanol ^a control 45 min	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
100 μM DCCD	CIAT899	0 ± 0 (100)	0.43 ± 0.08 (96.3)	0.02 ± 0.01 (93.4)
	CAP45	0.034 ± 0.001 (98)	0.29 ± 0.02 (95.8)	0.09 ± 0.04 (90.7)

^a Ethanol was used to solubilize inhibitors and was included in control cells

^b Percent reduction of P_i transport or ATP levels compared to controls are shown in parentheses (bold).

Exchange and Efflux of Phosphate

After dilution of pre-loaded cells in medium containing zero P_i or in media containing a 5-fold excess of unlabeled P_i , CIAT899 retained constant radioactivity, suggesting that neither P_i transport system will mediate P_i efflux or exchange internal P_i with external P_i (Fig. 5A and 5B). While somewhat irregular in response to efflux and exchange treatments, the mutant strain CAP45 behaved similarly (Figure 5C). CIAT899 cells loaded with 400 μM [^{32}P]PO₄ (evaluation of the low-affinity transport system) and diluted 100 times in zero- P_i medium maintained constant uptake of phosphate (Fig. 5B). After dilution there was enough [^{32}P]PO₄ in the medium to be taken up by the high-affinity transporter present in the wild type. In contrast, neither the high-affinity transport system of the wild-type (Fig. 5A) nor the low-affinity transporter of the mutant CAP45 (Fig. 5C) exhibited increased uptake under the same conditions because there was not enough phosphate after dilution to saturate either of these systems.

Discussion

Kinetic analysis showed that CIAT899 has two P_i transport systems; a high-affinity transporter and a low-affinity transporter. In contrast, CAP45 contains only a single P_i transport system that has low affinity properties. Further study is required to determine the exact genetic lesion in CAP45, however in analogy with *E. coli*, one reasonable speculation might be that

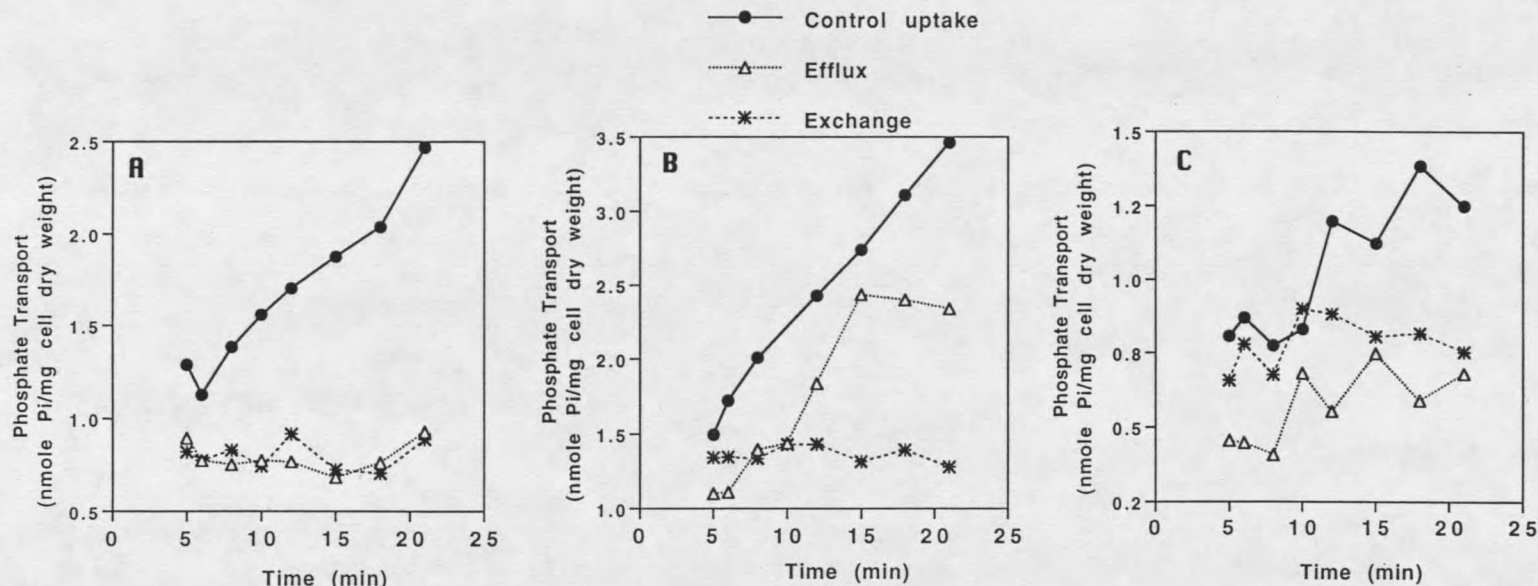


Figure 5. Absence of P_i exchange and efflux in *R. tropici* CIAT899 and CAP45. (A) -P_i cells of CIAT899 were loaded with 5 μM ³²P (specific activity = 22.5 μCi · μmol⁻¹) for 4 min, then diluted 100-fold in a medium containing 5 μM ³²P (uptake control); zero P_i (efflux) or 25 μM unlabeled P_i (exchange). -P_i cells of CIAT899 (B), and CAP45 (C) were loaded with 400 μM ³²P (specific activity = 22.5 μCi · μmol⁻¹) for 4 min, then diluted 100-fold in a medium containing 400 μM ³²P (uptake control); zero P_i (efflux) or 2 mM unlabeled P_i (exchange). Results are typical of two experiments documenting this response.

CAP45 is a mutant for the *pst* (high-affinity) system. This interpretation is consistent with the regulatory phenotype (constitutive expression of alkaline phosphatase) and the absence of the high-affinity transporter. However, polar effects on gene expression are commonly associated with transposon insertions and could complicate interpretations. If the transposon had interrupted a gene that is a negative regulator and that is promoter proximal in an operon with the gene coding for the membrane component of the permease, then lack of apparent transport via the high-affinity system could be due to lack of gene expression. On the other hand, a mutation in the gene coding for the permease protein could result in complete lack of transport. Regardless, the apparent lack of transport activity via the high-affinity system allowed for the characterization of the low-affinity system apart from other possible P_i -uptake pathways in the range of substrate employed in these assays.

The kinetic properties of the high-affinity system in *R. tropici* suggests that it is not atypical relative to high-affinity P_i transporters described in other bacteria (Willsky and Malamy, 1980; Van Veen *et al.*, 1993). However, the low-affinity system is atypical in that its K_m is much higher and indeed is more consistent with the range reported for secondary transport systems in other bacteria (Willsky and Malamy, 1980; Lacoste *et al.*, 1981; Van Veen *et al.*, 1993).

Kinetic analysis experiments with the wild type and mutant strains

showed osmotic shock drastically reduced maximum P_i transport velocities of both systems. Shock sensitivity was not observed with $+P_i$ cells, implying the P_i -binding protein is induced by P_i stress. This is consistent with the P_i -sensitive expression of *pstS*, the structural gene for the P_i -binding protein, in other Gram-negative bacteria. Other characteristics of these transport systems that suggest they belong to the traffic ATPase shock-sensitive class of permeases comes from experiments that showed P_i uptake by both systems was abolished when cellular ATP concentrations were decreased by 90% as a result of incubation with the ATPase inhibitor DCCD (Table 5). In contrast, neither system was substantially affected by the uncoupler CCCP, which was shown to dissipate membrane potential (Fig. 4), and which also strongly inhibits secondary transport systems (Berger and Heppel, 1974; Daruwalla *et al.*, 1981).

Efflux and exchange measurements also indicate that these P_i transport systems belong to the traffic ATPases class of transporters (Boos and Lucht, 1996). Movement of phosphate in *R. tropici* is unidirectional, from outside to inside, as no evidence of exchange and efflux was found (Fig. 5).

Summarizing, the data presented in this study demonstrate that *R. tropici* CIAT899 possesses two P_i transport systems. These transport systems differ in affinity for P_i by two orders of magnitude (Table 2), but are otherwise very similar. Both are inducible by P_i limitation (Table 2), are shock-sensitive (Fig. 3, Tables 3, 4), and apparently utilize ATP, or a derivative of ATP, to

energize the transport process (Table 5). The occurrence of two P_i transport systems in *R. tropici* is in contrast to a single P_i transport system reported for other rhizobia thus far examined (Smart *et al.*, 1984; Bardin *et al.*, 1996), and having two traffic ATPase primary transporters appears to be novel relative to other bacteria thus far studied (Rosenberg *et al.*, 1977; Lacoste *et al.*, 1981; Yashphe *et al.*, 1992; Van Veen *et al.*, 1993).

Functional duplication has been noted previously for *R. tropici*, and indeed reiteration is not uncommon for the Rhizobiaceae. *R. tropici* has been shown to contain two citrate synthases, one coded by a chromosomal gene and the other by a gene on the symbiotic plasmid (Hernandez-Lucas *et al.*, 1995; Pardo *et al.*, 1994); both citrate synthase enzymes are required for optimum symbiosis. Other examples of reiteration in the Rhizobiaceae include two glutamine synthetases in *B. japonicum* (Espin *et al.*, 1994) and three in *R. meliloti* (Espin *et al.*, 1994; Shatters *et al.*, 1993). *Mycobacterium tuberculosis* contains three genes that encode proteins closely related to *E. coli* PstS, the P_i -binding protein component of the high-affinity P_i permease system (Lefevre *et al.*, 1997). Together with the *M. tuberculosis* genes encoding homologs of the PstA, PstB, and PstC components (Braibant *et al.*, 1996), it was suggested that at least one, perhaps several, related and functional traffic ATPase phosphate transporters exist in mycobacteria (Lefevre *et al.*, 1997). These systems have not been characterized at the physiological level, and therefore it is not known if they are functional or if

they differ in their kinetic properties.

It is possible that one of the P_i transport systems found in *R. tropici* was acquired through horizontal gene transfer. In changing and challenging environments, bacteria must have the ability to acquire additional survival skills. Conjugation has played an important role in the evolution of antibiotic resistance in bacteria (Davies, 1994), and the potential for horizontal genetic exchange of pollutant-degradative genes exists in nature (Van der Meer *et al.*, 1992). CIAT899 was originally isolated from nodules of bean plants growing in extremely acid soils of Colombia, having soil solution P_i concentrations of less than 1 μM (P.H. Graham, personal communication). Therefore, the existence of two inducible P_i transport systems in *R. tropici* may be part of an adaptation response to an environment where P_i is always limiting. Having a dynamic genome may provide *Rhizobium* with a better possibility of adaptation to a changing environment under both free-living and symbiotic conditions.

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CHAPTER 4

ROLE OF THE HIGH-AFFINITY PHOSPHATE TRANSPORTER IN
Rhizobium tropici DURING SYMBIOSISIntroduction

Nitrogen fixation in the *Rhizobium*-legume symbiosis results from a complex interaction involving host, *Rhizobium*, and environment. If the requirements of the host and bacteria are not satisfied, the extent of nitrogen fixation and dry matter production of the plant are reduced. One nutrient requirement that has significant influences on the symbiosis is phosphorus. On a world-wide basis, soils are often low in phosphorus and thus this macronutrient frequently limits legume production. As compared to legumes grown nonsymbiotically with nitrogen fertilizer, legumes growing in symbiosis with *Bradyrhizobium/Rhizobium* have a higher phosphorus requirement (Israel, 1987), and phosphorus distribution within the legume changes (Sahrawat *et al.*, 1988). Responses to phosphorus fertilization include increased plant dry matter, shoot nitrogen content, nodulation, and nitrogen fixation, and has been documented for soybeans (Israel, 1987), common beans (Graham and Rosas, 1979; Pereira and Bliss, 1987, 1989), and cowpeas

(Cassman *et al.*, 1981).

Because phosphorus availability has a significant influence on this plant-microbe interaction, it is important to understand how phosphorus is metabolized by each symbiont and how phosphorus flows between partners. To accomplish this, it is relevant to study the type and level of phosphorus provided to the bacteroids. Bardin *et al.* (1996) have recently shown that inorganic phosphorus (P_i) is the form of phosphorus taken up by alfalfa bacteroids, however it remains to be determined whether phosphorus is first presented to the bacteroids as P_i or in an organic form that requires phosphatase activity to release P_i for uptake. Alkaline phosphatase is expressed at very high levels in *R. tropici* bacteroids (Al-Niemi *et al.*, 1997), suggesting organophosphate compounds may be important sources of phosphorus for bean bacteroids. In free-living *R. tropici*, alkaline phosphatase is not expressed unless media P_i levels are less than 1 μ M (Al-Niemi *et al.*, 1997). Therefore, bacteroid expression of alkaline phosphatase also implies the P_i concentration in the bean symbiosome space is quite low - in the K_m range of the high-affinity P_i transport system described in Chapter 3. The objective of this study was to test the P_i transport mutant CAP45 in symbiosis studies to determine if loss of the high-affinity P_i transport system would influence bacteroid P_i acquisition and/or overall effectiveness of the symbiosis.

Materials and Methods

Strains

Wild type strain *R. tropici* CIAT899 and the P_i transport mutant CAP45 were described and characterized in Chapter 3.

Phosphate Uptake Assay

P_i uptake was determined as described in Chapter 3.

Plant Culture and Symbiosis Studies

Seeds of *Phaseolus vulgaris* cv. Viva Pink were surface sterilized in 95% (v/v) ethanol for 10 sec, followed by treatment with 3% (w/v) sodium hypochlorite for 5 min., and rinsed 6 times with sterile deionized water. Surface sterilized seeds were germinated on yeast extract mannitol agar (Graham, 1963) for 48 h at 30°C. Seedlings showing no signs of contamination were transferred to growth pouches (Mega International of Minneapolis, MN, U.S.A.), previously sterilized by autoclaving at 121°C, 15 psi, for 1 h.

Initial watering of the growth pouches was with 50 ml of sterile nutrient solution prepared as described previously (McDermott and Kahn, 1992), except $FeCl_3 \cdot 6H_2O$ was replaced by an equivalent amount of iron supplied in a chelated form (Sprint 330, CIBA-GEIGY). Additional modifications to this nutrient solution included two P_i treatments, where the nutrient solution contained either zero phosphate ($-P_i$ plants) or 750 μM

K_2HPO_4 (+ P_i plants). In the - P_i treatment, plants received no exogenous P_i . The amount of P_i associated with the paper wick in the growth pouch was measured by adding 50 ml of either - P_i nutrient solution or 1 N HCl to test pouches, allowing 24 h to equilibrate, and then P_i assayed using the methods of Penney (1976). The P_i level in the 1 N HCl extraction was 208 μM , but the - P_i nutrient solution extraction was less than 1 μM , suggesting there was P_i associated with the paper wick, but that under conditions used in these experiments the amount of P_i available for plant use in - P_i treatments was minimal.

The plastic growth pouch is a closed system with respect to nutrient solution additions. Evapotranspiration results in salt accumulation because no nutrients are lost to leaching. In longer experiments such as those used here, salt toxicity was a concern. To determine which nutrient solution dilution resulted in optimum plant growth and avoided salt toxicity, the nutrient solution was diluted with distilled water to achieve dilutions of 1/2, 1/5, 1/10, and 1/20. Plants were cultured under conditions described below. Salt accumulation was determined by measuring the electrical conductivity of nutrient solution samples using an Accumet model 50 electrical conductivity meter.

For all growth pouch experiments, seedlings (one per pouch) were immediately inoculated with either CIAT899 or CAP45. The strains were grown to early stationary phase in MMNH_4 -10 mM P_i broth at 37°C

(containing $25 \mu\text{g} \cdot \text{ml}^{-1}$ gentamicin for CAP45), washed twice with $\text{MMNH}_4\text{-OP}$ to remove medium P_i and cell density adjusted to about $10^6 \text{ cells} \cdot \text{ml}^{-1}$. An additional set of uninoculated plants grown under $+\text{P}_i$ conditions was included to monitor contamination. Plants were grown in a growth chamber supplied with incandescent and fluorescent lighting providing approximately $400 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ during 16 h days. Temperatures were maintained at 24°C and 20°C for day and night, respectively. Nutrient solution was added weekly to maintain approximately 50 ml in each pouch. After 32 days, plants were harvested and acetylene reduction, nodule fresh weight and dry weight, nodule number, and shoot dry weight were determined.

Nitrogen fixation was estimated by acetylene reduction, using a method similar to that described by McDermott and Graham (1990). Immediately following shoot harvest, plant roots were transferred to a airtight 1 liter glass container outfitted with a rubber septum through which acetylene was injected (10% by volume). After 30 min of incubation, a 5 ml gas sample was withdrawn and stored in Vacutainer tubes (Becton Dickinson) until analyzed by gas chromatography. Nodules were removed and weighed, and plant shoots were dried at 65°C and weighed. Acetylene reduction activity (ARA) is expressed as $\mu\text{moles of ethylene (C}_2\text{H}_4) \text{ produced} \cdot \text{h}^{-1} \cdot \text{g of nodule fresh weight}^{-1}$.

The stability of the mutation in CAP45 during symbiosis was assessed

by testing nodule isolates for antibiotic resistance and P_i uptake rates. Fresh nodules were surface sterilized in 95% ethanol for 10 sec., 3% sodium hypochlorite for 3 min., and rinsed 6 times with sterile distilled water. The nodules were crushed in 0.85% (w/v) saline solution, serially diluted and plated onto $MMNH_4$ -10 mM P_i agar. After 3 days of incubation at 37°C, 25 isolated colonies were replica plated onto $MMNH_4$ -10 mM P_i and $MMNH_4$ -10 mM P_i containing 25 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin. For P_i uptake assays, randomly selected CAP45 nodule isolates were grown to early stationary phase in $MMNH_4$ -10 mM P_i broth (25 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin), washed twice with $MMNH_4$ -OP, and starved for phosphate for 7 h. P_i transport was then measured at 5 μM and 400 μM P_i as described in Chapter 3.

Bacteroid ^{32}P Accumulation *in situ*

In situ bacteroid P_i uptake was carried out as described by Al-Niemi *et al.*, (1997). After 32 days of culture in growth pouches, two plants per treatment were combined and transferred to 1 L beakers containing 600 ml of + P_i nutrient solution (750 μM PO_4) labeled with [^{32}P] KH_2PO_4 (specific activity = 0.45 $\mu\text{Ci} \cdot \mu\text{mol}^{-1}$). The entire root system was submerged into the labeled nutrient solution and then nodule samples taken after 30, 60, 90 and 120 min. At each time point, nodules were carefully removed, placed into 1.8 ml microcentrifuge tubes, and washed three times to remove label associated

with the nodule surface. For each wash, 1 ml of transport rinse buffer (described in Chapter 3), amended with 0.5 mM sodium arsenate (TRB-arsenate), was added to each nodule sample, vortexed 5 sec., and then the wash solution removed by vacuum. Nodules were crushed in the microcentrifuge tube containing 0.5 ml of fresh TRB-arsenate and then centrifuged at 12,000 $\times g$ for 5 sec to pellet nodule debris. A 250 μ l aliquot of the supernatant was carefully transferred to a fresh microcentrifuge tube, and centrifuged at 3,000 $\times g$ for 4 min. The supernatant was completely removed by vacuum, the pellet resuspended in 1 ml of TRB-arsenate and centrifuged again at 3,000 $\times g$ for 4 min. The supernatant was removed and the pellet was resuspended in 1 ml of TRB-arsenate. Al-Niemi *et al.*, (1997) demonstrated that this protocol resulted in isolation of bacteroids that were essentially free of plant organelles.

Aliquots of the resuspended final bacteroid pellet were used to determine optical density at 595 nm, which was converted to dry weight using the empirical equation $y = 0.803x - 0.006$, $r^2 = 0.99$; where y = mg bacteroid cell dry weight and x = optical density at 595 nm. To determine radioactivity in bacteroids, 750 μ l aliquots of the bacteroid suspension were collected by suction on 0.3 μ m glass fiber filters. The filters were placed in counting vials, 20 ml of scintillation cocktail (Scintisafe Plus™ 50%, Fisher Chemical) added, and ^{32}P was measured in a liquid scintillation analyzer.

Results

Free-living *Rhizobium tropici*

The high-affinity P_i transport defect in CAP45 was routinely verified prior to use of CAP45 in symbiosis experiments. Figure 6 shows typical P_i transport behavior of $+P_i$ and $-P_i$ CIAT899 and CAP45 cells. In these assays, the P_i concentration was 5 μ M, which is well below the K_m for the low-affinity P_i transport system, but saturates the high-affinity permease and readily distinguishes CAP45 as having a significantly reduced P_i transport rate. The increase in P_i transport observed with CAP45 $-P_i$ cells is due to induction of the low-affinity system discussed in Chapter 3.

Symbiosis Studies

Prior to examining the symbiotic performance of CAP45, nutrient solution concentration was optimized for the growth chamber temperature used and experiment duration. Electrical conductivity, a measure of salt accumulation, was less in treatments receiving more dilute nutrient solutions (Fig. 7A). After 26 days, severe burning at the leaf margins was evident on plants receiving full strength nutrient solution. Intervienal chlorosis was observed in plants receiving the 1/5, 1/10, and 1/20 dilution nutrient solutions, increasing in severity with the more dilute nutrient solution treatments. Half-strength nutrient solution resulted in highest plant dry matter production (Fig 7A). The effect of nutrient solution strength on

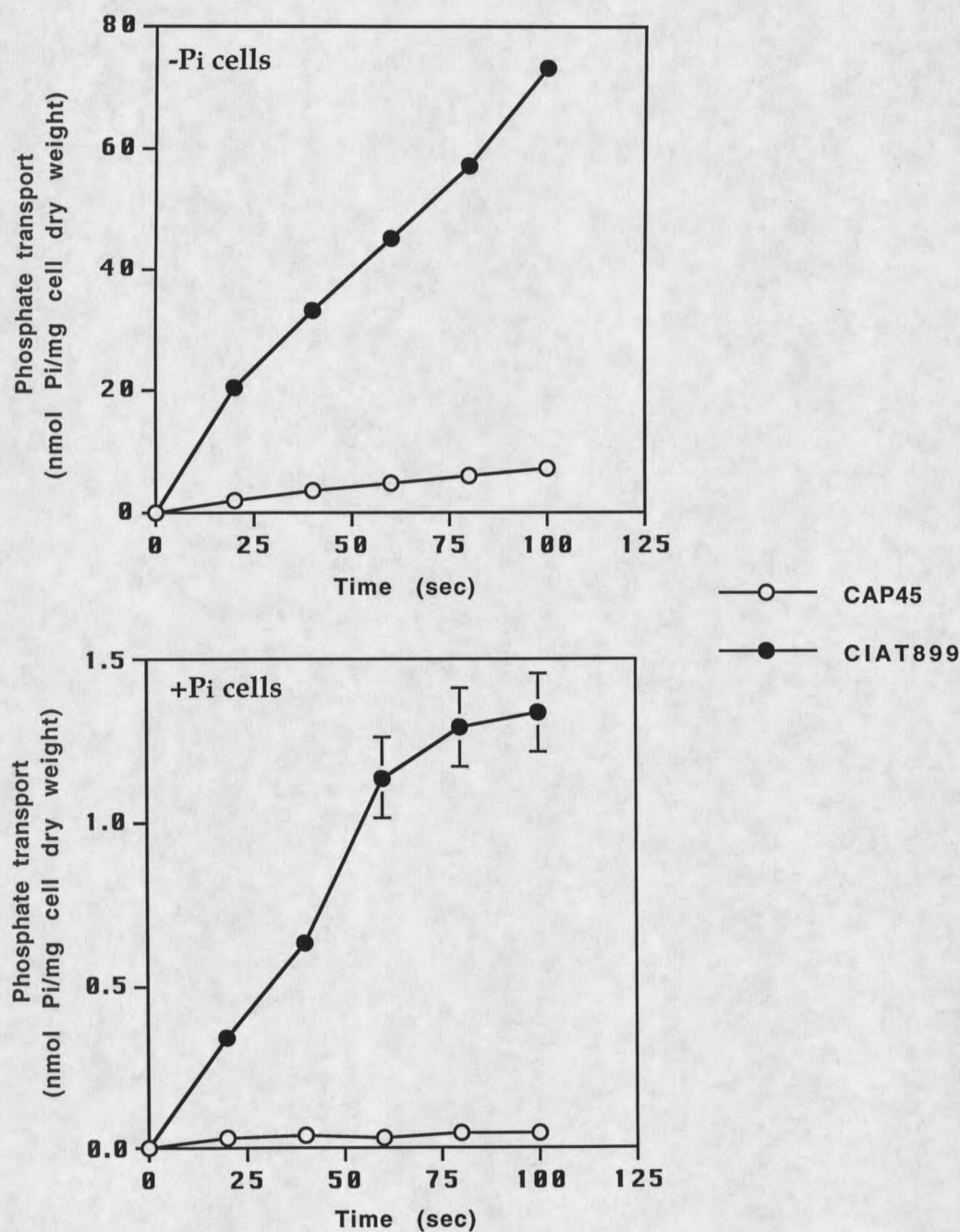


Figure 6. Phosphate uptake in *R. tropici* CIAT899 and CAP45. The transport assays and preparation of -P_i and +P_i cells was as described in Chapter 3. Assay starting P_i concentration was 5 μ M. Each point represents the mean of three replicate experiments. Error bars, where visible, represent the standard error.

nodule dry weight was similar, although greatest nodule weight occurred with plants receiving full-strength nutrient solution (Fig. 7B). From this experiment, it was concluded that 1/2 strength nutrient solution resulted in an optimum combination of plant growth and nodulation, and was used in subsequent experiments.

In experiments comparing the symbiotic phenotype of CAP45 to that of CIAT899, the influences of P_i stress on the symbiosis were evident (Table 6). For plants inoculated with either strain, shoot dry weight of $+P_i$ plants was approximately twice that of $-P_i$ plants, and nodulation and nitrogen fixation (acetylene reduction) were reduced 50% and 70%, respectively, by P_i -limitation (Table 6). Under $+P_i$ conditions, plants nodulated by the mutant CAP45 displayed significantly less shoot dry weight relative to plants nodulated by the wild-type strain (Table 6), however, no strain effects were observed with $-P_i$ plants. There were also no significant strain effects on nodulation or acetylene reduction.

No nodules were found on uninoculated controls, demonstrating that contamination was not a problem. Also, the transposon insertion in CAP45 was determined to be stable during symbiosis as 100% of the tested CAP45 nodules isolates were gentamicin resistant and lacked the high affinity P_i transport system (data not shown).

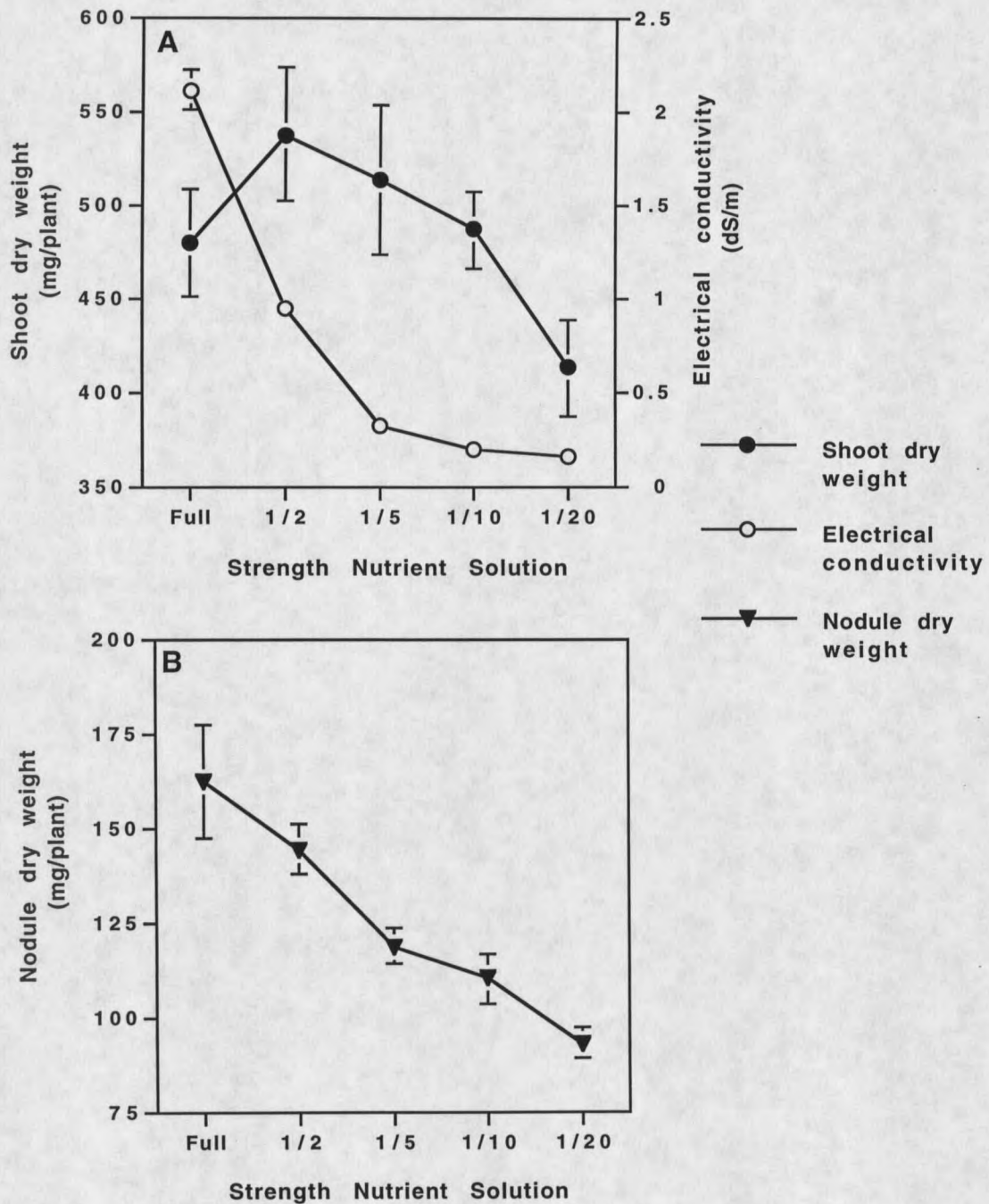


Figure 7. Effect of different strength nutrient solution (+P_i) on (A) shoot dry weight, salt accumulation and (B) nodule dry weight. CIAT899 was used as the inoculant, and plants were harvested 32 days after seedling transfer to the growth pouch. Data are from a single experiment, with five replicates for each nutrient solution treatment. Error bars represent the standard error.

Table 6. Symbiosis phenotype of the *R. tropici* wild-type strain CIAT899 and the high-affinity P_i transport mutant CAP45. Plants were grown for 32 days under $+P_i$ and $-P_i$ conditions as detailed in Materials and Methods. Data shown for plant dry matter is derived from three independent experiments and means for the other parameters are from two independent experiments. Means followed by the same letter in the same column are not significantly different. Each treatment was replicated 5 times in each experiment, and means were compared using an $LSD^a_{\alpha=0.05}$ derived from analysis of variance, using a block design where experiments were considered blocks.

P_i treatment and Strain	Shoot dry wt (mg/plant)	No. nodules per plant	Nodule dry wt (mg/plant)	ARA ^b ($\mu\text{mol C}_2\text{H}_4/\text{h/g nodule}$)
$-P_i$				
CIAT899	288 b	105 a	52.8 a	11.6 a
CAP45	254 b	118 a	68.9 a	11.8 a
$+P_i$				
CIAT899	651 d	183 b	184.7 b	34.3 b
CAP45	489 c	196 b	194.6 b	35.8 b
Not inoculated	104 a	0	0	0

^a LSD; Least Significant Difference

^b ARA; Acetylene Reduction Activity

Bacteroid ^{32}P Accumulation

To determine the extent that the high-affinity P_i transport system is involved in bacteroid P_i acquisition, *in situ* bacteroid P_i uptake was compared between CAP45 and CIAT899 in mature nodules of bean plants grown under $-\text{P}_i$ and $+\text{P}_i$ conditions (Fig. 8). For both strains, *in situ* bacteroid label accumulation uptake was significantly greater in $-\text{P}_i$ plants. Differences between CAP45 and CIAT899 were significant at all samplings in $-\text{P}_i$ plants, although the level of significance tested slightly lower ($\text{LSD}_{\alpha=0.10}$) at the 60 min, 90 min, and 120 min time points (Fig. 8A). In $+\text{P}_i$ plants, there were no differences in bacteroid P_i accumulation between CAP45 and CIAT899 during the first hour of labeling, but differences were significant ($\text{LSD}_{\alpha=0.05}$) at the last two sampling times (Fig. 8B).

Discussion

The nutrient solution described in this work has been used previously with beans grown hydroponically or in sand (Al-Niemi *et al.*, 1997). Plastic growth pouches were used in these experiments because they offered efficient use of plant growth space and provided for aseptic culture for extended periods. However, the design of the growth pouch does not allow for leaching of unused nutrients which can accumulate after repeated addition of nutrient solution. This is particularly a problem with larger legumes such as

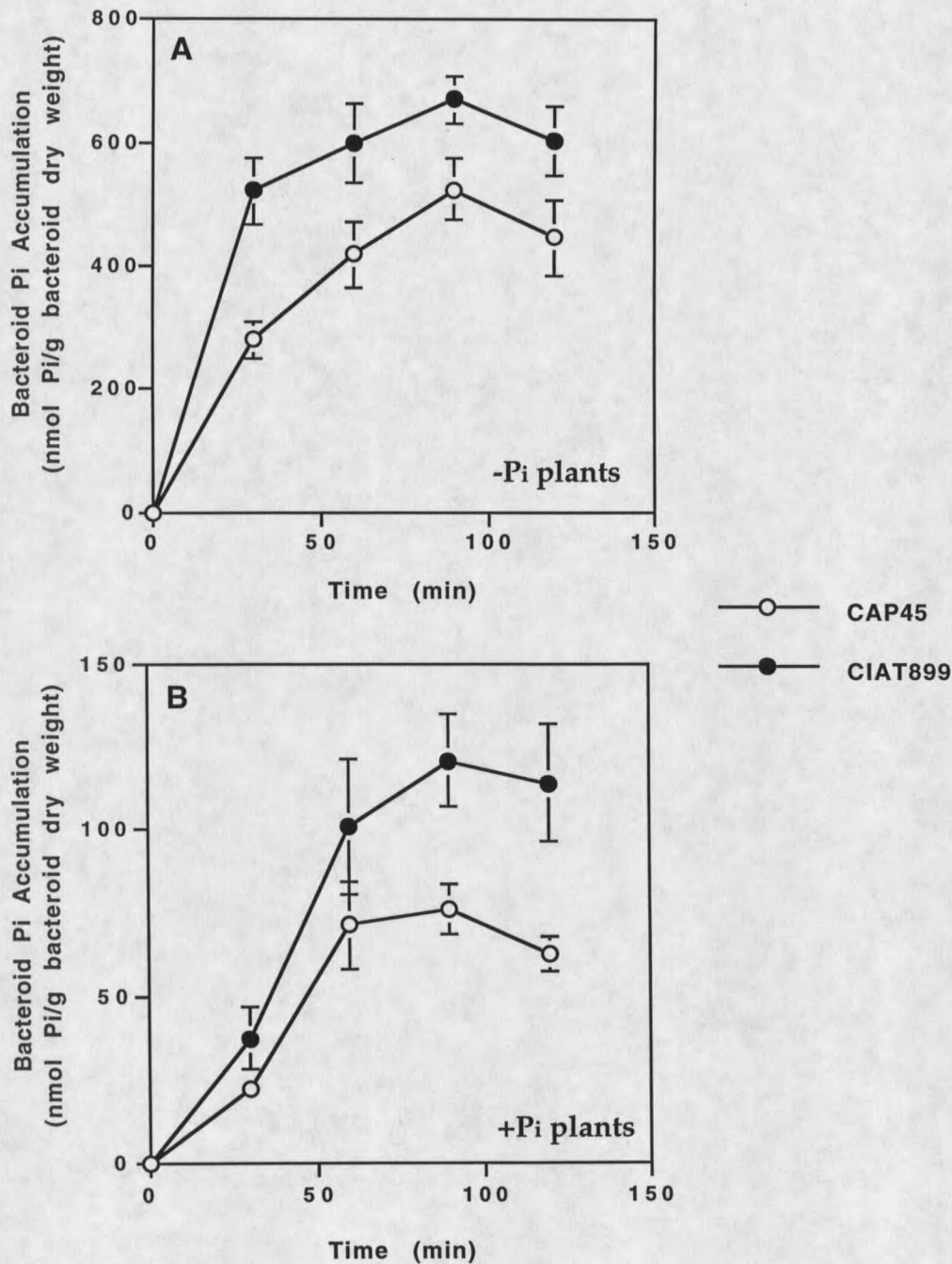


Figure 8. *In situ* CIAT899 and CAP45 bacteroid P_i accumulation. Plants were grown for 32 days under $-P_i$ (A) or $+P_i$ (B) conditions, and labeled with ^{32}P as described in Materials and Methods. Data is derived from two independent experiments. Each treatment was replicated 3 times in each experiment, and means were compared using an $LSD_{\alpha=0.05}$ derived from analysis of variance, using a block design where experiments were considered blocks.

bean, where rates of evapotranspiration are much greater than in smaller legumes of equivalent age (e.g. alfalfa). Similar problems can be encountered with Leonard jar assemblies (Vincent, 1970), where the surface area is even larger, providing for greater evaporation and higher risk of contamination. In comparisons of mutant phenotypes, there is zero tolerance of contamination. The optimum nutrient solution concentration for plant dry matter production and nodulation was found to be half-strength (Fig. 7). Electrical conductivity of the nutrient solution remaining in those pouches was $0.9 \text{ dS} \cdot \text{m}^{-1}$ and is similar to the optimum reported by Hall and Schwartz (1993). Repeated addition of full strength nutrient solution resulted in electrical conductivity of $2.1 \text{ dS} \cdot \text{m}^{-1}$ and symptoms that suggested salt toxicity. The electrical conductivity of more dilute solutions was lower, but beans receiving such solutions exhibited symptoms that implied sulfur or micronutrient deficiency (Hall and Schwartz, 1993).

The $-P_i$ treatment resulted in dry matter accumulation that was approximately half that of $+P_i$ plants. Half-maximal dry matter production indicated P_i stress and was used to compare the symbiosis responses of plants nodulated by the wild-type and mutant strains. Bean seeds contain significant amounts of P_i that can be used during the development of the symbiosis (Yan *et al.*, 1995). Initial application of even small amounts of P_i would delay the onset of P_i stress and require longer culture periods. Therefore, in order to

study the effects of the P_i -transport mutation under conditions where P_i was limiting for the host plant, no P_i was added to the $-P_i$ plants.

Al-Niemi *et al.* (1997) reported that *R. tropici* bacteroids express high levels of a P_i -stress-inducible alkaline phosphatase. In free-living *R. tropici*, this alkaline phosphatase is not induced until media P_i levels decrease to less than 1 μ M (Al-Niemi *et al.*, 1997). By analogy, this suggests the P_i concentration in the bean symbiosome space is in the K_m range for the high-affinity P_i transport system (Table 2, Chapter 3) and too low for the low-affinity P_i transport system to efficiently contribute to bacteroid P_i acquisition.

As measured by whole plant labeling in both $+P_i$ and $-P_i$ plants, *in situ* CAP45 bacteroid P_i uptake was only about 30% less than CIAT899 bacteroids (Fig. 8). By contrast, in experiments with free-living cells, P_i transport rates in CAP45 are less than 10% of the wild-type in either $+P_i$ or $-P_i$ cells (Fig. 6). These data imply that the high-affinity P_i permease accounts for a relatively small proportion of the total phosphorus flux into the bacteroid in mature nodules. Assuming the P_i concentration in the bean symbiosome space is indeed submicromolar, then the low-affinity P_i transport system would account for even a smaller proportion of total bacteroid P_i uptake. Therefore, provided *R. tropici* does not have an additional P_i transport system that is

expressed only in bacteroids, these observations suggests that organic phosphorus compounds may be the dominant form of phosphorus taken up by bean bacteroids.

CAP45 was found to nodulate and fix nitrogen to the same extent as the wild-type strain CIAT899. In symbiosis with $-P_i$ plants, the P_i transport mutation of CAP45 had no apparent effect on symbiosis. Any possible negative effect(s) may have been masked by the rather severe limitation imposed on the plant by the $-P_i$ treatment. By contrast, $+P_i$ plants nodulated by CAP45 accumulated 25% less dry matter than $+P_i$ plants nodulated by the wild-type strain. These results were consistently observed in three independent experiments. Assuming that CAP45 is isogenic to CIAT899, this implies the symbiosis phenotype of CAP45 is due to the inability of this strain to take up P_i through the high-affinity P_i transport system. While it has been argued that the phosphorus-sensitivity of the *Rhizobium*-legume symbiosis is a plant-controlled phenomenon (Jakobsen, 1985), the data reported here suggests that reduction of P_i supply to the bacteroids results in a similar reduction in nodule function efficiency.

There could be different explanations for the symbiosis phenotype of CAP45. Nodules need appropriate levels of solutes and water for metabolism and export of nitrogen fixation products (Walsh et al., 1989; Streeter, 1993). In plants nodulated by CAP45, it is possible that nodule and bacteroid solute

exchange is unbalanced due to the defective high-affinity P_i transport system, perhaps resulting in interrupted export of reduced nitrogen compounds. This is consistent with the observation that the deleterious effect of the P_i transport mutation was only observed in $+P_i$ plants, where adequate P_i would allow for a higher demand for reduced nitrogen and result in greater plant growth.

Factors other than P_i deficiency that might account for the symbiosis phenotype also need to be considered. As discussed above, the pleiotropic phenotype of CAP45 is consistent with the mutation being in a *pst* locus. In other Gram-negatives, the *pst* genes are arranged in operons and since the transposon insertion site in CAP45 has not been characterized, it is possible the symbiosis phenotype is partially complicated by the transposon having polar effects on expression of downstream genes coding for important symbiotic functions. Also, in addition to a P_i transport phenotype, CAP45 expresses alkaline phosphatase constitutively and so unregulated expression of alkaline phosphatase may itself be an issue. When combined with a nonfunctional high-affinity P_i permease, the hydrolysis of organic-phosphorus compounds could result in the release of P_i without an efficient mechanism available for uptake of that P_i . Furthermore, when phosphorylated, some carbon compounds (e.g. glycerol-3-P) are actively transported, whereas the nonphosphorylated version (e.g. glycerol) is taken in by passive diffusion. Hydrolysis of organic-phosphorus compounds could

result in reduced uptake of carbon compounds that are normally used as an energy source to fuel nitrogenase in *R. tropici*.

Consistent with previous studies (Al-Niemi *et al.*, 1997), *in situ* ^{32}P accumulation in bacteroids was significantly influenced by the P_i nutrition of the host plant. After 90 min of root labeling, bacteroid P_i accumulation in P_i -limited plants was six-fold greater than bacteroids in plants not subjected to P_i stress (Fig. 8). Al-Niemi *et al.* (submitted) have shown that differences in bacteroid label accumulation between $-\text{P}_i$ and $+\text{P}_i$ plants is not an artifact due to uptake of label by naked bacteroids during nodule processing. Therefore, the differences in *in situ* bacteroid P_i uptake between CAP45 and CIAT899 reflect actually bacteroid P_i accumulation and demonstrate the effects of the absent high affinity P_i permease in CAP45. Increased P_i accumulation in bacteroids of P_i -limited plants suggests that a mechanism for enhancing bacteroid P_i availability and uptake is in place under these circumstances. However, direct bacteroid participation in this phenomenon can be questioned because the increase in bacteroid label accumulation between $-\text{P}_i$ plants and $+\text{P}_i$ plants was roughly the same for CIAT899 and CAP45 bacteroids. Lack of P_i uptake through the high-affinity P_i transport system appeared to have little effect on this phenomenon, implying that the P_i -limitation effect is a predominantly plant-controlled function. Documented

plant responses to P_i stress include synthesizing acid phosphatases in roots and increased P_i uptake in tomato cell cultures suspension (Goldstein *et al.*, 1988; Lefebvre *et al.*, 1990; Hawkesford and Belcher, 1991), and so it is possible that P_i -stress to the host plant results in the induction of a plant permease located to the plant-derived peribacteroid membrane, resulting in enhanced transport of P_i or an organic phosphorus compound(s) across the peribacteroid membrane.

In the alfalfa symbiosis, a reasonably clear picture is emerging. *R. meliloti* has only a single functional P_i transport system (Smart *et al.* 1984; Bardin *et al.*, 1996), which when disabled results in the disruption of nodulation and a Fix^- symbiosis (Bardin *et al.*, 1996). This implies that while either organic-phosphorus compounds or P_i are provided to alfalfa bacteroids, P_i is the only form of phosphorus taken up by alfalfa bacteroids in quantities sufficient to support infection and normal levels of nitrogen fixation. Phosphorylated carbon compounds may be present in the symbiosome space, but either apparently are not taken up, or are not capable of being metabolized. In the bean symbiosis, phosphorus exchange between bacteroid and host requires the high-affinity system for a fully effective symbiosis. However further characterization of phosphorus exchange between the symbionts is needed. The role of the high-affinity system for phosphorus acquisition during symbiosis remains to be determined in the initial stages of

this process (i.e; bacterial multiplication) when there would be a higher demand of phosphorus for bacterial growth. Also, the importance of organic-phosphorus compounds or the low-affinity P_i transporter for symbiosis remain to be directly demonstrated. A *R. tropici* double mutant lacking both known P_i transport systems will be required to adequately address this question.

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CHAPTER 5

THESIS SUMMARY AND CONCLUSIONS

Agricultural production systems that are based on the philosophy of sustainability rely on practices that are ecologically sound and economically attractive. Incorporating legumes into crop rotations offers a means of reducing costly nitrogen fertilizer inputs, while maintaining economic return from the land. While legumes can reduce nitrogen fertilizer input requirements, they have specific phosphorus needs that are often not met by existing soil fertility levels. Advances in our understanding of phosphorus metabolism in the legume-*Rhizobium* symbiosis should enhance our ability to manage the phosphorus requirement of this important plant-microbe interaction and ultimately increase its agronomic potential. Current literature suggests responses of this symbiosis to phosphorus deficiency can be traced to effects on the host plant as well as nodule function. However, this symbiosis involves constant nutrient exchanges that involve both symbionts. Furthermore, very little is known about how bacteroids assimilate phosphorus and so it would seem inappropriate at this stage to conclude that bacteroid phosphorus metabolism has no consequences on nodule function.

As a first step towards a better understanding of bacteroid phosphorus acquisition, the specific questions addressed in this thesis research were:

- 1) How many P_i transport systems are functional in *R. tropici*?
- 2) Do bacteroids play a direct role in the well-documented phosphorus response?

In experiments designed to answer the first question, it was determined that *R. tropici*, unlike any other rhizobia thus far studied, contains two functional P_i transport systems. These P_i transport systems differ in their affinity for P_i by two orders of magnitude. *R. tropici* also appears novel relative to all other bacteria characterized at the physiological level in that both P_i transport systems are expressed constitutively, but are inducible by P_i stress. Based on physiological evidence, both transport systems are of the traffic ATPase class, having a periplasmic P_i -binding-protein component, and are energized by phosphate-bond energy. Neither transport system allows inorganic phosphorus efflux or exchange.

To answer the second question, a P_i transport mutant was tested in symbiosis studies to determine if loss of the high affinity P_i transport system would influence bacteroid P_i acquisition and/or overall effectiveness of the plant-microbe interaction. It was found that the high-affinity P_i transport system is required for a fully effective symbiosis when plants are growing under $+P_i$ conditions. Plants nodulated with the mutant strain showed

significant reduction in plant dry matter. Part of the defective function may be directly linked to reduced P_i uptake which was documented for bacteroids of this mutant. However, since the mutant still maintains a functional P_i transport system, a definitive answer regarding the relative importance of P_i as the primary source of phosphorus for bacteroids will require a double mutant that lacks both systems.

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