MONTANA STATE UNIVERSITY

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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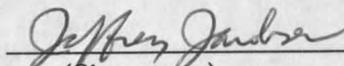
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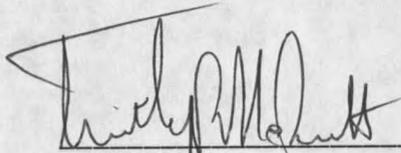
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This thesis is dedicated to my parents Rodrigo y Rubria and my husband Roger, because with their support, love, and encouragement I was able to accomplish my graduate studies.

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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 (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 *phoU*35 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 Vmax 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 Km (7 μM) for phosphate uptake in these bacteroids was higher than the Km (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
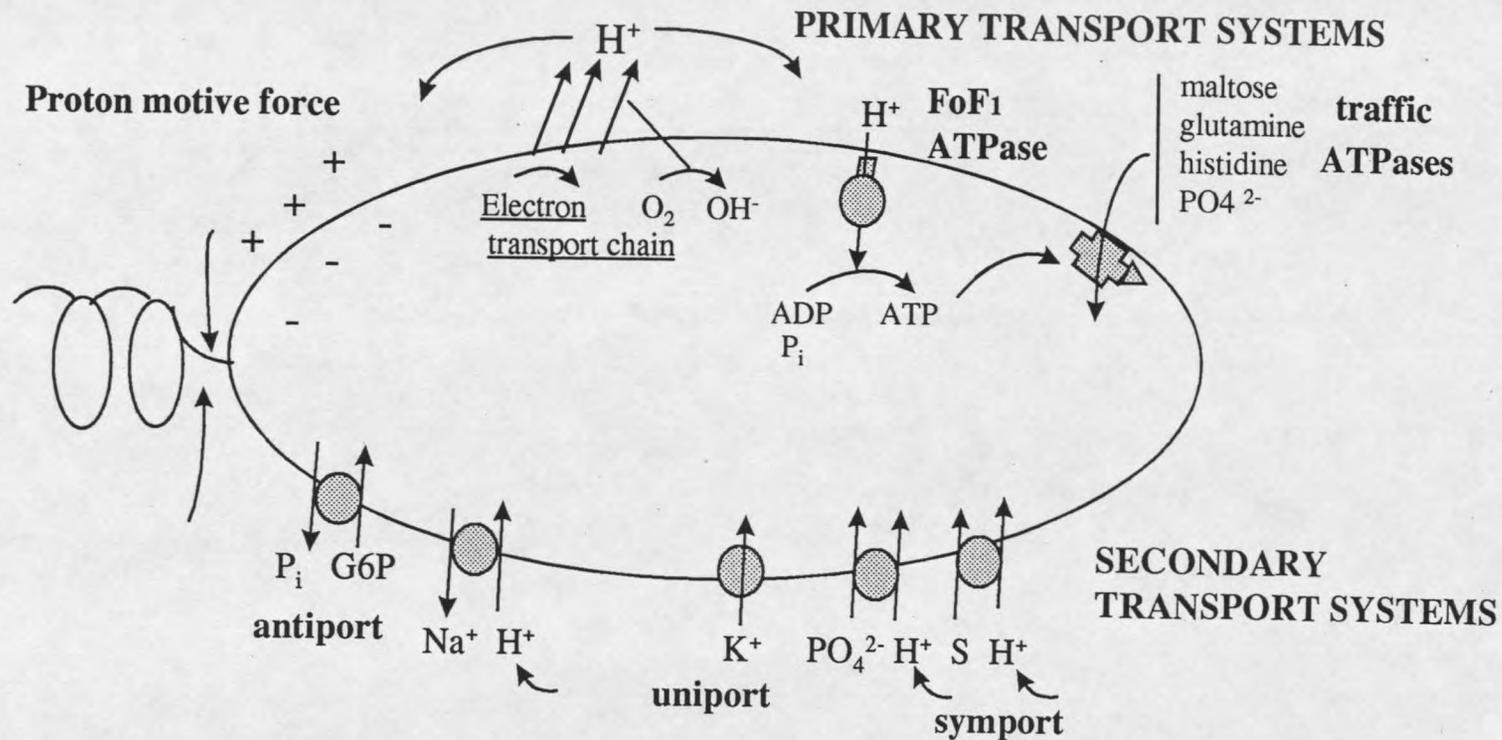
Figure 1. Solute transport in bacteria. The diagram illustrates generaton of a proton-motive force (Δp) by H^+ extrusion through the respiratory chain and use of Δp by several paths that perform work: FoF1-ATPase, the flagellar motor, and several kinds of secondary transport systems (proton-linked transport 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 traffic ATPases, 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 Km of 38 μM and a Vmax 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 (MeHPO4) 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 Km of 0.43 µM and a Vmax of 16 nmol $P_i \cdot min^{-1} \cdot$ 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 *ugp*B, *ugp*A, *ugp*E and *ugp*C form an operon. *ugp*B codes for the periplasmic binding protein for G3P, *ugpA* and *ugpE* code for the two membrane-bound components, and *ugp*C 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 μM.

When the concentration of P_i in the medium drops below 5 μM, 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*

Conditions	Substrate	Transport system	membrane transport genes	Affinity constant (μM)	Regulation
High P_i	P_i	Pit	*pit*	38	no
	G3P	GlpT	*glpT*	12	yes
	Hexose Phosphate	UhpT	*uhpT*	100	yes
Low P_i	P_i	Pst	*pstS, pstC, pstB, pstA*	0.43	yes
	G3P	Ugp	*ugpD, ugpB, ugpA, ugpC*	2	yes

Modified from Yagil (1987).

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