PHOSPHATE UPTAKE IN RHIZOBIUM MELILOTI

By

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Phosphate uptake in *Rhizobium meliloti*

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ABSTRACT

The soil bacterium *Rhizobium meliloti* fixes dinitrogen when associated with root nodules formed on its plant host *Medicago sativa* (alfalfa). A previously identified locus in *R. meliloti* 1021, *ndvF*, is required for nodule invasion and N$_2$ fixation. Genetic analysis of the locus revealed the presence of four genes, *phoCDET* which encode an ABC-type phosphate transport system. The transporter had a high affinity for phosphate (Km: 0.2µM) and took up phosphate with low velocity (Vmax: 6.7nmol/min/mg protein). It also showed a low specificity for phosphate as it likely transports phosphonate compounds as well. Expression of transcriptional *lacZ* fusions to *phoD* and *phoE* revealed that these genes were induced upon phosphate limitation and required a functional PhoB protein for their expression. *phoCDET* mutants grew poorly in MOPS-buffered minimal media containing 2mM Pi as sole phosphorus source, and failed to transport phosphate at concentrations usually found in the soil (1-10µM). This suggested that the symbiotic phenotype associated with mutation in this locus was the result of poor growth in this environment.

Analysis of two classes of mutation that suppressed the Fix$^-$ phenotype of *phoCDET* mutants confirmed the function of this Pi transporter and provided insights into its regulation. Sequence analysis of the ClassI (*sfx1*) suppressor
locus revealed the presence of two genes orfA and pit which likely form an operon. While orfA did not share any significant homology with proteins found in the Data Bank, pit showed homology to phosphate transport systems of various organisms, including the pit gene of Escherichia coli. The sfx1 mutation appeared to be a thymidine deletion in a hepta-thymidine sequence located 80 nucleotides upstream of orfA. orfA pit expression increased in a sfx1 background (compared to the wild type) leading us to hypothesize that suppression by sfx1 occurred by increasing Pi uptake by the Pit (OrfA) phosphate transport system.

The class II suppression mutations (sfx2 and sfx3 alleles) were unable to express the alkaline phosphatase enzyme (AP) suggesting that the mutations probably affect the Pho regulatory genes of R. meliloti. Three AP$^-$ Tn5-132 mutants linked in transduction to sfx2 were isolated. Sequence analysis of the DNA flanking these insertions revealed that they were located within the phoU and phoB genes of R. meliloti. These phoU and phoB mutations were able to suppress all the phenotypes associated with phoCDET mutations in a manner similar to sfx2. PhoB was also shown to be required for the uptake and/or metabolism of phosphonate compounds and activation of phoCDET expression. On the other hand, the product of the phoB gene, either directly or indirectly, negatively regulated pit expression in a phosphate dependent manner (i.e. repression under low phosphate conditions and in the phosphate starved environment of phoCDET mutant strains). Suppression of the symbiotic defect of
phoCDET mutants by phoUB (sfx2) mutations appeared then to occur by derepressing the pit system of *R. meliloti*, allowing increased phosphate uptake via this transporter.
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LIST OF ABBREVIATIONS

AEP
AMP
Ap
AP
ARA
Cm
EP
EPS
G3P
Glc6P
Gm
kb
Km
MP
min
Nm
NPP
OD
ONPG
ORF
Ot
P
P0
Pi
PSer
RT
SE
Sm
Sp
Tc
Rif
sec
vol
XGal
X-Phos

aminoethylphosphonate
aminomethylphosphonate
ampicillin
alkaline phosphatase
acetylene reduction activity
chloramphenicol
ethylphosphonate
exopolysaccharide
glycerol-3-phosphate
glucose-6-phosphate
gentamicin
kilobase pairs of DNA
kanamycin
methylphosphonate
minute(s)
neomycin
p-nitrophenyl phosphate
optical density
o-nitrophenyl-β-D-galactopyranoside
open reading frame
oxytetracycline
phosphorus
no phosphate added
inorganic phosphate
phosphoserine
room temperature
standard error
streptomycin
spectinomycin
tetracycline
rifampicin
second (s)
volume
5-bromo-4-chloro-3-indolyl-β-D-galactoside
5-bromo-4-chloro-3-indolyl phosphate
CHAPTER I

Introduction

A- The Rhizobium-legume symbiosis

Although gaseous nitrogen (N₂) constitutes 80% of our atmosphere, its inert nature makes it unavailable to eukaryotic organisms. The reduction of nitrogen gas to ammonia, known as nitrogen fixation, is however widely distributed among eubacteria and archaebacteria. Depending on the organism, nitrogen fixation occurs either in free-living state or in association with an eukaryotic organism such as in the Rhizobium/legume symbiosis. This symbiotic interaction between microorganism and eukaryotic host is agronomically important as it enables the plant host to grow in a nitrogen limited environment, thus reducing the requirement for nitrogen fertilizer.

This section reviews the different steps leading to the establishment of an effective symbiosis between Rhizobium bacteria and legume plant, focusing on the contribution of the bacterial symbiont.
All steps of nodule (plant organ in which the symbiosis takes place) development involve the expression of nodule-specific plant genes called nodulins (van Kammen, 1984). The early nodulin genes encode products that are expressed before the onset of nitrogen fixation and are involved in infection and nodule development. The products of the late nodulin genes are involved in the interaction with the symbiont and the metabolic specialization of the nodules (Nap and Bisseling, 1990). Analysis of the plant genes involved in root-nodule development was recently reviewed by Franssen et al. (1995) and will not be described in this literature review.

1) *Rhizobium* classification

Nitrogen-fixing, legume root nodule bacteria are gram-negative soil bacteria classified in the alpha subdivision of Proteobacteria (Holt et al., 1994). They are currently classified into three genera: *Rhizobium* ("fast grower"), *Bradyrhizobium* ("slow growers") and *Azorhizobium* ("stem nodulating") which so far has one characterized species that forms nodules on the stems of *Sesbania* (Dreyfus et al., 1988). *Bradyrhizobium* has just one named species as well; isolates that nodulate soybean (*Glycine max*) are called *Bradyrhizobium japonicum* whereas those that do not are known as *Bradyrhizobium* sp. followed by the host genus.
The named species of *Rhizobium* are *R. leguminosarum*, *R. meliloti*, *R. loti* (Jarvis et al., 1982), *R. fredii* (Scholla and Elkan, 1984) and *R. galegae* (Lindström, 1989). *R. leguminosarum* has been divided into three groups to reflect the host specificity (or biovar) defined by the plasmid carried (Jordan, 1984). The groups are clover (bv. *trifolii*), bean (bv. *phaseoli*) and pea, vetch and lentil (bv. *viciae*). *R. meliloti* strains, separated into two distinct genetic groups, nodulate alfalfa (*Medicago sativa*) and related legumes (Eardly et al., 1990).

The use of molecular techniques such as 16S ribosomal DNA comparisons, gene sequences analysis, DNA hybridization etc., showed that *Rhizobium* and *Bradyrhizobium* are not closely related (Young, 1992); *Rhizobium* resembles the plant tumor-inducing *Agrobacterium* more than it does *Bradyrhizobium* and *Bradyrhizobium* is phylogenetically related to the phototropic *Rhodospseudomonas palustris*. *Azorhizobium* is closer to *Bradyrhizobium* than to *Rhizobium* and is closely related to *Xanthobacter*. These data are supported by biochemical and physiological characteristics such as: in *Rhizobium* and in the closely related *Agrobacterium*, the nature of the plant interaction is determined by plasmid-borne genes, while in *Bradyrhizobium* and *Azorhizobium* there is no evidence for plasmids carrying symbiotic genes. Both *Bradyrhizobium* and *Azorhizobium* are able to fix nitrogen asymbiotically (Dreyfus et al., 1988).
II) Infection and nodulation

1- The Nod factors

The Rhizobium signal molecules which play a key role in the induction of the initial stages of nodulation are lipochito-oligosaccharides known as Nod factors. The nod genes involved in the synthesis of Nod factors are not expressed in free-living state bacteria with the exception of nodD, which is expressed constitutively. Upon binding to specific flavonoids secreted by the root of the plant host (Goethals et al., 1992), NodD activates transcription of the other nod genes (Fisher and Long, 1992). The structure of the major Nod factor of *Rhizobium meliloti* was determined by Lerouge et al. (1990) as a 6-O-sulfated N-(C\(_{16:2}\)) acyl-tri-N-acetyl-\(\beta\)-1,4-D-glucosamine tetrasaccharide. In general, Nod factors consist of a backbone of three to five \(\beta\)-1,4-linked N-acetylg glucosamines bearing a fatty acid on the non-reducing sugar residue, the synthesis of which is catalyzed by the products of the nodABC locus. The backbone is further modified by the action of other Nod proteins that synthesize or add various substituents. These substituents determine host specificity as well as the biological activity of the molecules (for detailed information on Nod factor structures and biosynthesis see Dénarié and Cullimore, 1993; Carlson et al., 1995). *Rhizobium meliloti* interacts with only a limited number of plant hosts and thus produces only a few different Nod factors (Truchet et al., 1991). In contrast,
Rhizobium NGR234, which can nodulate various tropical legumes, secretes 18 different Nod factors (Price et al., 1992).

Purified Nod factors were shown to induce root hair deformation at concentrations as low as \(10^{-12}\)M (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993; Heidstra et al., 1994). Nod factors also induce the expression of certain plant genes encoding early nodulins (Horvath et al., 1993; Jourmet et al., 1994; Cook et al., 1995).

2- Infection

After attachment of rhizobia to the root hair tips (Smit et al., 1992), the tips curl tightly and bacteria become "entrapped" within the curls. A local hydrolysis of the plant cell wall takes place in the curled region (Van Spronsen et al., 1994), the plasma membrane invaginates and new plant cell wall material (probably containing some nodulins) is deposited. This forms a tubular structure, the infection thread, by which the bacteria enter the plant (see Kijne, 1992 for review).

Simultaneously with infection thread formation, some cortical cells are mitotically reactivated and form the nodule primordium (Kijne, 1992). Nod factors are sufficient to induce this mitotic reactivation (Spaink et al., 1991; Truchet et al., 1991) probably by changing the phytohormone (auxin/cytokinin) balance.
(Long, 1996; Copper and Long, 1994; Hirsch et al., 1989) via the induction of yet unidentified early nodulin genes. Infection threads grow toward this primordium and once they reach the cortical cells, bacteria are released into the cytoplasm.

Lipopolysaccharides of *R. leguminosarum* bv. *trifolii* (Dazzo et al., 1991) and exopolysaccharide of *R. meliloti* (Dylan et al., 1986; Niehaus et al., 1993) appear to be required for the formation of the infection thread.

Beside infection through root hairs, which is the most widely studied and the one observed when *R. meliloti* infects its plant hosts, rhizobia can also enter through cracks in the epidermis (i.e. symbiosis between *A. caulinodans*/*Sesbania rostrata*; Dreyfus and Dommergues, 1981; Ndoye et al., 1994; *Bradyrhizobium* sp./*Parasponia andersonii*; Trinick, 1988) or at the junction of epidermal cells (route of entry of rhizobia in *Mimosa scabrella*; de Faria et al., 1988).

3- **The nodule**

The infection route as well as the structural and developmental characteristics of an efficient nodule are determined by the plant host (Rolfe and Gresshoff, 1988; Dénarié et al., 1992) with two types of legume nodules distinguished; indeterminate and determinate (Newcomb, 1981). Both types possess peripheral vascular bundles and a central tissue which contains infected and uninfected cells. Indeterminate nodules can be divided into specific zones which characterize a certain developmental stage from the distal meristematic
zone to the proximal senescence zone (Vasse et al., 1990). The meristem is followed by the prefixation zone, where infection takes place, the zone III, containing the differentiated, nitrogen-fixing bacteroids, and the senescence zone where bacteria are degraded by the plant. Thus in these cigar-shaped nodules, growth and functioning occur simultaneously and all stages of differentiation can be observed in a single longitudinal section. In determinate nodules, bacteria infect the meristematic cells. The nodule meristem ceases to divide at an early stage of development so that all the cells of the central tissue differentiate simultaneously to form a nitrogen fixing tissue (Newcomb, 1981). In this case, nodule growth and function are dissociated, the nodules are round shaped and only a single stage of bacterial differentiation can be observed at any given time.

4- **Metabolite exchange between plant and microsymbiont**

Upon release from the infection thread, the bacteria become internalized by a process resembling endocytosis. The microsymbiont is surrounded by a membrane derived from the host plasma membrane, referred to as the peribacteroid membrane (PBM). The PBM has phospholipid and protein compositions that are different from the plasma membrane (Perotto et al., 1995; Verma, 1992). It also contains several nodulins as well as rhizobial proteins (Verma and Hong, 1996; Fortin et al., 1985). Because the PBM constitutes the
interface between bacteroids and the plant host, it plays an important role in controlling the exchange of metabolites. These include ammonium, the product of nitrogen fixation (O'Gara and Shanmugan, 1976) which is exported from the bacteroid to the host cytoplasm, while the host supplies the bacteroid with dicarboxylic acids as carbon source (Ronson et al., 1981; Yarosh et al., 1989; Werner, 1992) which are derived from the sucrose synthesized in the plant leaves (Hawker, 1985). It was speculated that the heme prosthetic group of leghemoglobin was synthesized by the bacteria and exported to the plant host. Recent work however suggested that heme was synthesized by the plant (for review see O'Brian, 1996). The form in which nitrogen is transported depends upon the plant: legumes forming determinate nodules import ureides, whereas legumes forming indeterminate nodules import amides after the ammonium is assimilated in the cytoplasm of nodule cells via the glutamine synthetase/glutamate synthase pathway (Schubert, 1986).

5- **Nitrogen fixation**

Symbiotic nitrogen fixation in bacteroids is catalyzed by the nitrogenase enzyme following the reaction:

\[ \text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{Mg-ATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{Mg-ADP} + 16\text{Pi} \]

Nitrogenase consists of two components, the homodimeric Fe protein, encoded by *nifH*, and the tetrameric molybdenum-iron (MoFe) containing protein, encoded
by *nifD* and *nifK*. The MoFe cofactor is irreversibly denatured by oxygen, which makes nitrogenase highly sensitive to oxygen (Shaw and Brill, 1977). On the other hand, there is a high demand for oxygen in nodules as the large amount of energy required for the nitrogenase reaction is generated by oxidative processes. In legume nodules, the low oxygen tension in the central part of the nodule is achieved by a combination of a high metabolic activity and an oxygen barrier in the periphery of the nodule (i.e. the nodule parenchyma; Witty et al., 1986). In the infected cells the oxygen carrier leghemoglobin facilitates oxygen diffusion.

The *nif* genes encoding enzymes, involved in the nitrogen fixation process, are suddenly induced in the interzone II-III of the nodule. From extensive studies on *nif* gene regulation, it is found that these genes are induced under microaerobic conditions (Fischer, 1994; Soupène et al., 1995). Low oxygen is sensed by FixL in *R. meliloti* which activates the FixJ protein by phosphorylation upon microaerobiosis. Phosphorylated FixJ, in turn induces the transcription of *nifA* and *fixK* (Galinier et al., 1994; Reyrat et al., 1993). Both genes encode transcriptional activators of *nif* and *fix* genes, the products of the latter genes being involved in biochemical functions associated with nitrogen fixation (Cebolla and Palomares, 1994).
III) *Rhizobium meliloti*

*Rhizobium meliloti* strains form nitrogen-fixing nodules on species of only three genera: *Medicago*, *Melilotus* and *Trigonella*. *R. meliloti* possess two large extrachromosomal replicons, referred to as megaplasmids. The presence of the two megaplasmids of 1200 to 1700kb in size appear to be a general feature among *R. meliloti* strains (Banfalvi et al., 1985; Bromfield et al., 1987; Burkhardt and Burkhardt, 1984; Burkhardt et al., 1987; Hynes et al., 1986; Finan et al., 1986; Rosenberg et al., 1982). In *R. meliloti* SU47, the two megaplasmids have been designated pRmeSU47a and pRmeSU47b. Both plasmids contain genes required for efficient nitrogen fixation; megaplasmid pRmeSU47a carries the *nod* genes and some *nit* and *fix* genes involved in nodule induction and nitrogen fixation (David et al., 1987; Debelle et al., 1986; Banfalvi et al., 1981; Rosenberg et al., 1981) whereas the larger megaplasmid pRmeSU47b carries genes involved in exopolysaccharide and thiamine biosynthesis (Finan et al., 1986; Glazebrook and Walker, 1989; Zhan et al., 1989; Glucksmann et al., 1993), dicarboxylic acid transport (Finan et al., 1988; Watson et al., 1988) as well as genes required for lactose (Charles et al., 1990), dulcitol, melibiose, raffinose, β-hydroxybutyrate, acetoacetate, protocatechuate and quinate utilization (Charles and Finan, 1991). Defined deletions of megaplasmid pRmeSU47b allowed the isolation of a new symbiotic locus, initially designated *fix-114* (Charles and Finan,
1991). Analysis of this locus constitutes the subject of the work presented here and will be discussed in greater detail later in this literature review.
B- Phosphate uptake in microorganisms

1) In *Escherichia coli*

*Escherichia coli* possesses two major inorganic phosphate (Pi) transporters; the Phosphate Specific Transporter (Pst) and the Phosphate Inorganic Transporter (Pit). Pst, a high affinity low-velocity system (Km: 0.4μM and Vmax: 15.9 nmol of Pi per min per mg of protein) is induced under conditions of phosphate limitation (Willsky and Malamy, 1980a). In contrast, Pit is a low affinity, high-velocity system (Km: 38.2μM and Vmax: 55 nmol of Pi per min per mg protein) and is constitutively expressed. In addition, two organophosphate transport systems (GlpT and UhpT) as well as the phosphonate transport system (PhnCDE) are capable of taking up phosphate as a secondary substrate (Ambudkar et al., 1986a; Metcalf and Wanner, 1991).

1- The Pst system

The Pst system is part of the Pho regulon. Its expression is inhibited when the preferred P source, Pi, is in excess and is induced 100 fold (or more) when the environmental Pi has been exhausted (Wanner, 1996). This transport system is a periplasmic protein-dependent transporter belonging to the superfamily of ABC (ATP-binding cassette) transport systems which are also called traffic ATPases. These type of transporters are present in a wide variety of organisms.
including mammals (Higgins, 1992). The Pst system is composed of two integral membrane channel proteins PstA and PstC, PstB, a membrane bound ATPase protein believed to be active as a dimer (Chan and Torriani, 1996) and PstS the periplasmic phosphate-binding protein. Genes of the Pst components are arranged in an operon along with a protein called PhoU; the pstSCAB-phoU operon (Surin et al., 1985). PhoU, a peripheral cytoplasmic protein, has no effect on Pi uptake but may be involved in the overall process of Pi assimilation via the Pst system. Steed and Wanner (1993) observed that the low growth phenotype associated with a ΔphoU mutation was alleviated in a ΔpstB-phoU or ΔpstSCAB-phoU mutation, suggesting that the accumulation of Pi taken up by the Pst transport system in the absence of PhoU led to growth inhibition. In addition, both PstSCAB and PhoU are required for repression of the Pho regulon under phosphate sufficient conditions. The “repression function” of the PstSCAB complex is independent of its transport function (Cox et al., 1988). The mechanism of phosphate transport via the Pst system has been studied using site directed PstA, PstC and PstS mutants. The proposed model is as follows: the PstS protein, highly specific for phosphate, sequesters the monovalent or divalent phosphate anions from the periplasmic space and transfers it to the first membrane protein-binding site upon interaction of PstS with PstA and PstC. The transfer of phosphate to the second membrane protein-binding site and its subsequent release into the cytoplasm occurs as a result of conformational
changes energized by ATP hydrolysis which is catalyzed by the PstB subunits (see Webb and Cox, 1994 for review).

2- The Pit system

Pit is a single-component proton motive force-driven transporter analogous to LacY (Kaback, 1990), also called chemiosmotic carrier. Van Veen et al. (1994b) demonstrated that the substrate for the Pit system reconstituted in proteoliposomes was not inorganic phosphate but rather a neutral metal phosphate ($\text{MeHPO}_4$) chelate, formed by Pi complexing with divalent metal ions (such as $\text{Mn}^{2+}$, $\text{Mg}^{2+}$, $\text{Ca}^{2+}$, $\text{Co}^{2+}$). A homologous transport mechanism was also characterized for the second Pi transport system of *Acinetobacter johnsonii* (Van Veen et al., 1993b). Besides uptake of metal phosphate, Pit also mediates efflux and homologous exchange of metal phosphate in the absence of a driving force (Van Veen et al., 1994b). The fact that Pit (1) utilizes $\text{MeHPO}_4$ as a substrate, (2) is not required for growth with Pi as sole phosphorus source and is not involved in the Pho regulon control, raises questions about the physiological role of the Pit system. Pit may actually be a divalent metal transporter for which Pi is an effective anion (Van Veen et al., 1994b).

Genetic analysis of the *pit* locus is just beginning. Cells with a functional Pit protein were shown to be arsenate sensitive, due to the fact that arsenate is a substrate for this transporter. Using this phenotype *pit* mutants were isolated as
arsenate resistant (Willsky and Malamy, 1980b). All known pit mutations are mapped within the same locus (77 min) of the E. coli chromosome. The Pit locus has been cloned (Elvin et al., 1986) and its sequence contains a single open reading frame that encodes a 499 amino acid polypeptide (Sofia et al., 1994).

3- The organophosphate uptake systems

Like Pit, GlpT and UhpT are chemiosmotic carriers. The GlpT transport system is very similar to the UhpT transporter both at the amino acid sequence level of the proteins and transport mechanism (Eiglmeier et al., 1987; Ambudkar et al., 1986a). GlpT is part of the glp regulon which is induced by the presence of glycerol-3-phosphate (G3P) and repressed by GlpR (Larson et al., 1987; Lin and Iuchi, 1991). glpT and glpQ (encoding a periplasmic phosphodiesterase (Lin, 1976)) constitute an operon which maps near min 49 on the chromosomal map of Escherichia coli. The induction of uhpT transcription, in response to extracellular hexose-phosphate such as glucose-6-phosphate (Glc6P) (Dietz, 1976), is regulated by the uhpABC genes. These genes are located immediately upstream of uhpT at 82.1 min on the E. coli genetic map (Weston and Kadner, 1988). Both GlpT and UhpT accumulate G3P and sugar-phosphate, respectively, in exchange for internal phosphate. These systems are then capable of heterologous (G3P:Pi), (Glc6P:Pi) exchanges as well as homologous (Pi:Pi) exchanges, with Pi acting as competitive inhibitor for the uptake of the
organophosphates (Ambudkar et al., 1986a; Maloney et al., 1990). Whether the GlpT and UhpT system contribute to Pi uptake under conditions of Pi limitation has not been examined. Net Pi uptake by these anion exchangers may result from modification of the stoichiometric exchange between the organophosphate compound and Pi in response to intra or extracellular environment. This was observed for glucose 6-phosphate uptake in an analogous transporter of *Streptococcus lactis* (Ambudkar et al., 1986b).

G3P can also be taken up by the Ugp system (uptake of glycerol phosphate) in response to phosphate limitation (Argast and Boos, 1980). The Ugp system is a periplasmic binding protein-dependent transporter specific for G3P and glycerol phosphoryl phosphodiesters, (deacylation products of phospholipids; Argast, 1978). G3P transported by the Ugp system can only be used as phosphorus source in contrast to the G3P transported by GlpT system which can be used as both carbon and phosphorus source. G3P taken up by the Ugp system is not utilized by a specific pathway, it simply enters the common G3P pool with a rapid incorporation into phospholipids as well as proteins (Schweizer et al., 1982). The inability of G3P to serve as carbon source when transported by the Ugp system appears to be the result of uptake inhibition of this transporter by internal Pi accumulation as a result of G3P metabolism (Xavier et al., 1995).
4- The phosphonate transport system

Phosphonates (Pn) are organophosphorus compounds with a direct carbon-phosphorus bond. They can be found in various organisms (see Jiang et al., 1995 for review) as constituents of glycolipids, glycoproteins, polysaccharides or phosphonolipids. Industry also introduces synthetic phosphonates to the environment such as the herbicide glyphosate. Bacteria have evolved two pathways for uptake and degradation of phosphonates in order to use these compounds as sole phosphorus sources. These pathways utilize different mechanisms of C-P bond fission. The phosphonatase pathway is specific for phosphonates in which one hydrogen on the second carbon of the molecule has been substituted (such as 2-amino-ethylphosphonate) while the C-P lyase pathway has a broad substrate specificity. A given organism may have one or both pathways: *E. coli* for example has only the C-P lyase pathway (Wanner and Boline, 1990), *Salmonella typhimurium* carries genes for the phosphonatase pathway only (Jiang et al., 1995) while *Enterobacter aerogenes* carries genes for both pathways (Lee et al., 1992).

The genes encoding the C-P lyase pathway proteins of *E. coli* are organized in a single operon made of 14 genes (*phnC to phnP*) (Metcalf and Wanner, 1993). The *phnCDE* genes probably encode a binding protein-dependent (ABC type) Pn transporter with PhnD as the periplasmic binding protein, PhnE an integral membrane protein and PhnC the ATPase component
of the transport system. \textit{phnF} and \textit{phnO} appear to encode regulatory proteins while genes \textit{phnG} to \textit{phnN} probably encode proteins required for cleavage of the C-P bond (see Wanner, 1996 for review). Results from growth studies with various mutants suggested that Pi as well as phosphoserine are taken up by the PhnCDE transporter as non-specific substrates (Metcalf and Wanner, 1991). Recently, a global analysis of proteins synthesized from cells grown in the presence of phosphonates as sole phosphorus source revealed the induction of 227 proteins and the repression of 30 (out of 816 proteins monitored). 137 of these 227 proteins were also regulated by Pi limitation (118 induced, 19 repressed; VanBogelen et al., 1996).

5- The Pho regulon

Genes induced under Pi limitation and transcriptionally activated by the PhoB protein constitute the phosphate (Pho) regulon. In \textit{Escherichia coli} more than 30 genes belonging to this regulon have been cloned and sequenced. They are arranged as eight separate transcriptional units and are involved in the uptake and assimilation of phosphorus compounds from the environment. In addition to the \textit{pst-phoU}, \textit{ugp}, and \textit{phn} operons described above, the Pho regulon includes the following genes: The Pho regulatory genes, \textit{phoBR} (Makino et al., 1986a and b); \textit{phoA} which encodes a periplasmic alkaline phosphatase enzyme (AP) involved in the hydrolysis of organophosphates (Coleman, 1992);
phoE which encodes an outer membrane porin (Overbeeke et al., 1983); and phoH which encodes an ATP-binding protein whose function in vivo is yet to be characterized (Kim et al., 1993). A recent study, analyzing proteins synthesized during phosphorus limiting condition (using two-dimensional (2-D) polyacrylamide gel electrophoresis) suggested that 413 proteins out of the 816 studied were induced (208 proteins) or repressed (205 proteins) when the cells were grown under phosphate limitation (VanBogelen et al., 1996). This result is in fact not surprising if we consider phosphate as being a central component in many metabolic pathways. Analysis of these proteins will also probably lead to the discovery of new genes belonging to the Pho regulon.

a) Pi-dependent control of the Pho regulon

The primary control of the Pho regulon is the Pi concentration of the media. The work of Rao et al. (1993) suggested that it is the external phosphate concentration that regulates the Pho regulon and not the cytoplasmic Pi concentration. $^{31}$P-NMR measurements revealed that the intracellular Pi concentration of 9 to 13mM, when extracellular Pi is in excess, remains high (about 7mM) during derepression of the Pho regulon (i.e. when the cells are grown under phosphate limitation; Rao et al., 1993).

Pi control of the Pho regulon involves two processes: Pho activation when Pi is limited and Pho inhibition when Pi is in excess.
Activation of the Pho regulon occurs via a two-component regulatory system (Parkinson, 1993) consisting of the transmembrane sensor histidine kinase, PhoR, and the transcriptional activator PhoB. When the external phosphate concentration falls below 4μM, PhoR undergoes autophosphorylation on a histidine residue using ATP as the phosho-donor. It then promotes PhoB phosphorylation on an aspartate residue (Makino et al., 1989) via its kinase activity. Phosphorylation of PhoB enhances its binding activity to the Pho Box, an 18 nucleotide sequence [5'-CT(T/G)TCATA(A/T)A(T/A)CT(T/G)TCA(C/T)-3'] consisting of two direct repeats of 5'-CT(T/G)TCAT-3' that flank a A+T-rich 4bp spacer. The Pho Box is located 10 nucleotides upstream from the putative -10 region in the promoter of the genes activated by PhoB (Wanner, 1996). No sequences homologous to the -35 region has been found in these promoters. Pho boxes are tandemly repeated twice and 3.5 times in the regulatory region of pstS and ugpB, respectively (Wanner, 1993). Mutational analysis of the pstS promoter region indicates that each Pho Box is functional (Kimura et al., 1989).

Inhibition of the Pho regulon requires, a high external phosphate concentration, the PhoU protein, an intact (although not necessary able to transport phosphate) Pst system, the phosphate specific transporter, and PhoR. PhoR is believed to dephosphorylate phospho-PhoB when the external Pi concentration increases above 4μM. As a small region of the PhoR protein is exposed to the periplasmic space (Scholten and Tommassen, 1993), sensing of
the external phosphate signal may be mediated by Pst and PhoU. Excess phosphate in the media may lead to the formation of a “repression complex” in which the PhoR phosphatase activity is induced. When the external Pi concentration decreases, the low Pi occupancy of PstS (or PhoR) may provoke conformational changes in the Pst complex and/or PhoU leading to the release of PhoR. PhoR then is activated by autophosphorylation which enhanced its kinase activity and thus phosphorylates PhoB (see model Fig. 1-1).

i) The PhoB protein

The PhoB protein is a cytoplasmic protein composed of 229 amino acids containing at least three domains: I- phosphorylation domain; II- DNA-binding domain (that recognizes the Pho Box); and III- domain that interacts with the RNA polymerase holoenzyme (Wanner, 1996). Truncated PhoB and point mutants were used to identify these domains (Makino et al., 1989; 1994 and 1996). Domain I is located in the N-terminal 127 amino acids with Asp-53 as the phospho-accepting residue and Thr-83 that appears to play an important role in the phosphate transfer reaction. The C-terminal 90 amino acids of PhoB constitutively activate transcription from the Pho genes suggesting that both domain II and III are located in this area of the protein. It is not known whether domain III is physically separable from domain II or overlaps it. Analysis of rpoD mutants (encoding the σ^{70} subunit of RNA polymerase), that are specifically
Transmembrane signal transduction by environmental $P_i$. Notation: PhoB, response regulator; PhoB-P, phospho-PhoB; PhoR$^A$, PhoR activation (autophosphorylated) form; PhoR$^R$, PhoR repression (inhibition) form; PhoU, phoU gene product; PstA and PstC, integral membrane channel proteins of Pst transporter; PstB, traffic ATPase of transporter; PstS, periplasmic $P_i$-binding protein; small triangles, $P_i$ binding site on PstS and a hypothetical $P_i$ regulatory site on PhoR.

Fig. 1-1: Phosphate regulation of the Pho regulon of *E. coli* as proposed by Wanner (1996).
defective in the expression of the pho genes, suggests that PhoB directly interacts with the first helix of the putative helix-turn-helix motif in the C-terminal region (region 4.2) of $\sigma^{70}$ (Makino et al., 1993). $\sigma^{70}$ likely contacts four amino acids of the PhoB protein located around the turn connecting $\alpha$-helix 2 (amino acids 176 to 187) and 3 (amino acids 189 to 207), helices as deduced from their homology with the histone H5 structure (Makino et al., 1996). It was then proposed that the PhoB/$\sigma^{70}$ interaction permits the RNA polymerase to enter the pho promoters for initiation of transcription. A recent study showed that a region around amino acids 87-97 of PhoB may be critical for interactions with histidine kinases (Haldimann et al., 1997).

ii) The PhoR protein

The PhoR protein is composed of 431 amino acids. Membrane topology studies, using phoR-phoA fusions and deletion mutants, suggested that PhoR is anchored to the cytoplasmic membrane by two transmembrane domains (N-terminus of the protein) (Scholten and Tommassen, 1993). PhoR exerts both positive and negative regulation of the Pho regulon. The positive regulation is due to its kinase activity, demonstrated in vitro, that leads to PhoB phosphorylation (Makino et al., 1989). The negative regulation is believed to occur by dephosphorylation of PhoB by PhoR; however the phosphatase activity of PhoR is yet to be demonstrated. Isolation of mutants constitutive for either
activation or repression of the Pho regulon suggests that the two activities are separable from one another. Since the two types of mutations were generally mapped in close proximity and intermixed locally, the functional elements should be intimately interacting with each other to allow reversible functional changes. The histidine His-213 is conserved among histidine kinases. Moreover, constitutive repression of the Pho regulon by point mutation (H213Y) strongly suggests His-213 to be the site of autophosphorylation in PhoR (Makino et al., 1992).

b) **Pi-independent control of the Pho regulon**

The Pho regulon is subject to multiple positive controls due to the fact that once Pi is taken up by the cells it can be incorporated into ATP via several central pathways. These pathways include: oxidative phosphorylation (via the ATP synthase enzyme), glycolysis (via the glyceraldehyde-3-phosphate dehydrogenase and the phosphoglycerate kinase enzymes), tricarboxylic acid cycle (via the succinyl coenzyme A synthetase) and mixed-acid fermentation (via the phosphotransacetylase/acetate kinase pathways).

At least two Pi-independent controls act on the Pho regulon in the absence of PhoR. CreC (formerly PhoM) a protein kinase, like PhoR, phosphorylates PhoB as well as CreB a transcriptional activator that regulates the expression of unknown genes (Amemura et al., 1990). The other Pi-
independent control requires acetyl phosphate which may directly phosphorylate the PhoB protein (Wanner, 1992).

Evidence for activation of the Pho regulon by the CreC protein was provided by the isolation of mutants in a phoR<sup>−</sup> background that completely abolished Pi control of the Pho regulon. Mutation in creC led to a 400-fold induction in alkaline phosphatase (AP) synthesis. This level was however lower than the level of AP synthesized in the presence of PhoR during phosphate limitation (1000-fold induction) (Wanner et al., 1988). This induction was abolished in a phoR creC double mutant suggesting that CreC was responsible for the induction.

The discovery of acetyl phosphate as an effector of the Pho regulon arose with the isolation of AP<sup>+</sup> revertants from a phoR creC double mutant. These pseudorevertants carried mutations in the ackA gene (for acetate kinase) that led to accumulation of acetyl phosphate. Because of severe growth defects, the triple mutants rapidly reverted to AP<sup>−</sup> through mutations in the pta gene (phosphotransacetylase) (Wanner and Wilmes-Riesenberg, 1992). Acetyl phosphate is an intermediate of the phosphotransacetylase-acetate kinase (Pta-AckA) pathway. Acetyl phosphate is made from acetyl-CoA and Pi by Pta and degraded into acetate and ATP by AckA. Mutations or growth conditions leading to increased levels of acetyl phosphate synthesis, either directly (i.e. growth on
pyruvate as carbon source) or indirectly, led to activation of the Pho regulon (Wanner and Wilmes-Riesenber, 1992).

It is interesting that an intermediate in Pi metabolism (incorporation of Pi into ATP) seems to be involved in Pho regulon control probably via the [ATP] / [acetyl phosphate] ratio, with a lowered ratio causing induction. By extension, Pi-independent control by the CreC protein may be coupled to a different central pathway of carbon and energy metabolism (for entry of Pi into ATP). Such controls may provide a basal level of expression of the Pho regulon when the system is Pi repressed which could be especially important in wild-type strains undergoing shifts of carbon and energy sources.

II) Phosphate uptake in other micro-organisms

1- In *Pseudomonas aeruginosa*

In *Pseudomonas aeruginosa*, Pi limitation results in the synthesis of several proteins, including alkaline phosphatase (AP), an outer membrane protein P, as well as a hemolytic and nonhemolytic phospholipase C (Hancock et al., 1990; Shortridge et al., 1992). Pi transport in *P. aeruginosa* occurs with biphasic kinetics, suggesting the involvement of both a high affinity (*pst*-like) with an apparent Km of 0.46 ± 0.1μM and a low-affinity (*pit*-like) transporter with a Km of 12.0 ± 1.6μM (Poole and Hancock, 1984). The nucleotide sequence of the
phosphate-transport system was recently determined and revealed the presence of four open reading frames, \textit{pstC}, \textit{pstA}, \textit{pstB} and \textit{phoU} that probably constitute an operon (Nikata et al., 1996). The \textit{pst} operon was Pho-regulated as indicated by the presence of a well conserved Pho Box preceding \textit{pstC} and the 25 fold induction in level of expression in the presence of a functional PhoB protein. The deduced PstC and PstA proteins are both larger than their \textit{E. coli} homologues with an additional hydrophilic domain in the N-terminal part of the protein. The most striking difference with the \textit{pst} operon of \textit{E. coli} is the absence of the \textit{pstS} gene, which encodes the periplasmic Pi-binding protein. A Pi-binding protein required for Pi uptake via the high-affinity transport system has been purified (Poole and Hancock, 1984), suggesting the gene encoding this protein is not part of the \textit{pst} operon in \textit{P. aeruginosa}. Under phosphate limitation, \textit{P. aeruginosa} shows a chemotactic response towards Pi (Kato et al., 1992) which may be important for scavenging Pi under these growth conditions. Both PstB and PhoU (but not PstC and PstA) are essential to repress Pi taxis and alkaline phosphatase under conditions of Pi excess (Nikata et al., 1996) but unlike AP expression, Pi taxis does not require the \textit{phoB} and \textit{phoR} gene products for regulation (Kato et al., 1994, Nikata et al., 1996).
2- In *Acinetobacter johnsonii*

*Acinetobacter johnsonii* 210A accumulates excessive amounts of phosphate as polyphosphate (polyP) under aerobic conditions. Pi is taken up by two phosphate transport systems similar to the high- (pst) and the low-affinity (pit) systems of *E. coli*. The high affinity transport system of *A. johnsonii* belongs to the group of ATP-driven binding protein-dependent transport systems. It is induced 6-10 fold by Pi limitation and has a Km value of $0.7 \pm 0.2 \mu M$. The low-affinity uptake system is constitutively expressed, is inhibited by uncouplers and has a Km of $9 \pm 1 \mu M$ (Van Veen et al., 1993a). In *A. johnsonii*, Pi uptake is monophasic (over time) in contrast to the biphasic uptake of Pi in *E. coli* (Medveczky and Rosenberg, 1971), *P. aeruginosa* (Poole and Hancock, 1984) and *Bacillus cereus* (Rosenberg at al., 1969). Biphasic uptake is explained by the presence of two transport systems, with the high affinity transport system subject to inhibition when a primary pool of Pi within the cells is filled. This prevents accumulation of the solute to unacceptably high internal levels. Because Pi taken up by *A. johnsonii* is rapidly converted into polyphosphate, a low internal phosphate concentration can be maintained and Pi uptake remains linear until a maximum level is reached. Metal-phosphates (MeHPO$_4$; with Me being divalent cations such as Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Co$^{2+}$) rather than Pi is transported by the secondary phosphate transport system, while the primary Pi transporter mediates the uptake H$_2$PO$_4^-$ and HPO$_4^{2-}$ only (Van Veen et al.,
1994a). Stoichiometric studies suggest that the translocation of a (neutral) metal-phosphate together with one proton occurs via an electrogenic mechanism (Van Veen et al., 1993b). Anaerobic conditions lead to polyphosphate degradation in A. johnsonii followed by excretion of MeHPO₄ via the secondary transporter that was also shown to mediate MeHPO₄ efflux under these conditions. The two Pi transporters of A. johnsonii therefore evolved to allow the organism to efficiently use the predominant Pi species available. Genetic study of the Pi transport systems of A. johnsonii has yet to be reported.

3- In Bacillus subtilis

In the framework of the Bacillus subtilis genome sequencing project, genes homologous to the pst transporter of E. coli were characterized (Takemaru et al., 1996). The deduced amino acid sequence of the five ORFs of the pst locus of B. subtilis are similar to pstS, pstC, pstA and pstB. The fifth gene is also homologous to pstB so that two pstB genes (58% identity) are tandemly repeated while no phoU gene is present. These five genes likely form an operon. The presence of two Pho Boxes upstream of pstS suggests that these genes are under Pho regulation. The primary Pho regulon regulatory proteins, encoded by the phoP and phoR genes, constitute a two component regulatory system (response regulator and histidine kinase, respectively) similar to the PhoB and PhoR proteins of E. coli (Seki et al., 1987; 1988). The operon shows low level of
expression during vegetative growth and is induced during phosphate limitation. The Pho regulon of *B. subtilis* is also regulated by two other pairs of two-component regulatory systems. The *resD/E*, which affects respiratory-enzyme induction, is part of the Pho regulon and is also required for full induction of the *phoPR* operon. On the onset of sporulation, the response regulator encoded by *spoA* represses the Pho regulon via the AbrB protein probably by acting on the *phoPR* transcription (Hulett, 1995).

Phosphate transport systems have also been described in *Streptococcus lactis* (Poolman et al., 1987), *Streptococcus faecalis* (Harold et al., 1965; Harold and Banda, 1966; Harold and Spitz, 1975), *Micrococcus lyzeodeikticus* (Friedberg, 1977), *Bacillus cereus* (Rosenberg et al., 1969) and *Anabaena variabilis* (Thiel, 1988).

III) *Phosphorus in Rhizobium spp.*

Several studies have shown that *Rhizobium* and *Bradyrhizobium* species as well as their serotypes vary greatly in their tolerance to low levels of phosphate, their ability to store polyphosphates during growth under phosphate sufficient conditions and the utilization of these reserves for subsequent growth (Beck and Munns, 1984; Smart et al., 1984 (b); Cassman et al., 1981(a)). Delayed growth was observed when these cells were grown in a low phosphate
environment. Many rhizobial strains are, however, able to grow at nearly normal growth rates with phosphate concentrations commonly found in soils (0.1 to 10μM; Bieleski, 1973; Cassman et al., 1981b). It is interesting that many *R. meliloti* strains require a high calcium concentration in soil to grow at the low P levels found in soil solution (Beck and Munns, 1984; 1985).

Growth in a low phosphate soil environment suggests that Rhizobium strains evolved very efficient phosphate transport systems (Cassman et al., 1981b; Smart et al., 1984b). In this respect, Smart et al. (1984a) analyzed the effect of P nutrition on phosphate uptake in seven rhizobial and bradyrhizobial strains. They observed that phosphate-limited cells took up phosphate 10 to 180-fold faster than phosphate-sufficient cells. The apparent Km value for the phosphate uptake system was similar in all strains ranging from 1.6μM to 6.0μM phosphate with a Vmax from 17.2 to 126 nmol min⁻¹ (mg dry weight of cells)⁻¹. Phosphate uptake in all strains was inhibited by carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and these transporters failed to catalyze exchange or efflux of phosphate. The authors concluded that these strains carried a single, repressible, unidirectional and energy-dependent phosphate transport system. Alkaline phosphatase (AP) activity was strongly induced in *Rhizobium* strains in response to limiting phosphate and repressed under phosphate-sufficient conditions. No AP activity was detected in several *Bradyrhizobium* strains regardless of the P-status of the environment (Smart et
al., 1984a and b), suggesting that this may be a general phenomenon amongst bradyrhizobia.

Plants dependent on nitrogen fixation have a higher P requirement for optimum nitrogen accumulation than those utilizing combined nitrogen (Israel, 1987). The growing nodule is a major phosphate reservoir in the plant with P concentrations in the millimolar range (Mosse et al., 1976). This is probably due to the very high demand for ATP of the nitrogenase enzyme and the higher P content of the microbial tissue than plant cells. Even under phosphate limitation, the nodule remains the plant organ with the highest phosphate content. Using $^{31}$P NMR spectroscopy, Rolin et al. (1989) showed that Pi and P metabolites were not uniformly distributed in the different cell types of soybean nodules. Phosphate deficiency often limits dinitrogen fixation of legume-Rhizobium interactions due to reduction in both nodule numbers and mass. However, the roles of P in nodulation and nodule activity remain unclear. Sa and Israel (1991) and Israel (1993) suggested that the decrease in $N_2$ fixation resulting from P deficiency was both a direct effect on nodule metabolism and an indirect effect due to growth reduction of the plant host.

$P$ deficiency does not significantly alter the number of bacteroids per unit of nodule mass, but does decrease the bacteroid dry mass per unit of nodule dry mass (due to either decrease in cell size or density) and their nitrogen content (probably due to the inhibition of protein synthesis) (Sa and Israel, 1991). These
authors also observed that under P deficiency the adenylate energy charge (defined as \( \frac{[\text{ATP}] + \frac{1}{2} [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \)) and the ATP concentration were decreased in plant nodule cells but not in the bacteroids. In addition, the acetylene reduction activity (ARA) per nodule mass was not reduced (Ribet and Drevon, 1995a) and the ratio of total nitrogen fixed relative to nodule mass was not affected by P deficiency (Pongsakul and Jensen, 1991). This suggests that P deficiency limits total nitrogen fixation by inhibiting energy-dependent reaction(s) in the plant cells of the nodules rather than directly inhibiting nitrogenase activity.

Earlier papers (Bethenfalvay and Yoder, 1981; Robson et al., 1981; Jakobsen, 1985; Sa and Israel, 1991; Israel, 1993) mentioned a decrease in nitrogenase activity upon phosphorus limitation. This discrepancy with Ribet and Drevon’s results was explained by the different procedure used for the acetylene reduction assay (open-flow assays on whole plants that was less disturbing to the system than detopped root systems), and the assumption made by the earlier authors that the acetylene-induced decline (AID) in ethylene production rate (Minchin et al., 1986) was the same under all growth conditions. Ribet and Drevon (1995b), however, recently demonstrated that AID increases with P deficiency thus leading to a reduced ARA for P deficient compared to P sufficient plants.
Upon alleviation of phosphorus deficiency, the rapid phosphate increase in the shoots and the nodules occurs nearly simultaneously (Israel, 1993). This is followed by an increase in nodule growth indicated by nodule mass and ARA, which coincides with increase in the nitrogen content of these two plant organs. So, enhanced acetylene reduction upon alleviation of phosphorus deficiency may be associated with both increased carbohydrate supply from the leaves (photosynthesis was reduced under phosphate deficient conditions (Clarkson et al., 1983)) and/or enhanced carbohydrate utilization within the nodules. Nitrogen concentration in the stems and roots increases after delay followed by an increase in plant dry weight.

Delayed nodulation is observed when Pi-starved cells and cells with lower tolerance to phosphate limiting conditions are used to inoculate their host plants (Mullen et al., 1988; Leung and Bottomley, 1987). This delay may primarily be the result of a reduction in bacterial density in the rhizosphere of the plant root. However growth per se may not be the determining factor for successful nodulation. McKay and Djordjevic (1993) observed that excretion (rather than production) of Nod factors in *R. leguminosarum* was decreased under low phosphate conditions and that the extent of this decrease was strain dependent. So growth efficiency and Nod factor excretion may not necessarily be related. Low external phosphate concentration was also responsible for reducing the attachment of *R. meliloti* to roots of annual medics (Howieson et al., 1993).
Several studies reported that phosphate limitation stimulates the biosynthesis and/or alters the structure of rhizobial cell surface carbohydrates such as exopolysaccharide II (Zhan et al., 1991) and cyclic β-(1,2)-glucans in *R. meliloti* (Breedveld et al., 1995) and lipopolysaccharide in *R. leguminosarum* (Tao et al., 1992). As these compounds are required for effective symbiosis, their modification, under phosphate limiting conditions, may have profound effects on the infection of the plant host.
C- Previous work on ndvF and sfx

1) The ndvF locus

Testing strains containing defined deletions of the pExo megaplasmid for their symbiotic phenotype on plants led to the identification of the symbiotic locus ndvF (formerly fix114; Charles and Finan, 1991; 1990; Fig. 1-2a). The two overlapping deletions of the megaplasmid carrying this locus, Δ5408 and ΔF114 which delete about 150kb, also remove a region known to carry the exp genes (involved in the biosynthesis of EPS-II) (Glazebrook and Walker, 1989) and a previously unidentified locus, exoZ, which is involved in the synthesis of the acidic exopolysaccharide succinoglycan (EPS-I) of R. meliloti. Neither the exp biosynthetic genes nor exoZ was associated with the symbiotic (Fix') phenotype of the deletions. Rm5408 and RmF114 as well as a 12kb deletion of ndvF and Tn5 insertions in the locus, all induced small white nodules containing very few bacteria when inoculated on alfalfa plants, indicating that the infection is blocked at an early stage. This type of nodule is structurally similar to nodules formed by exopolysaccharides (exo) and β-(1,2)-glucans (ndv) mutants (Finan et al., 1986; Dylan et al., 1986), thus the designation ndvF for nodule development.

The ndvF locus was isolated after complementing the RmF114 Fix' phenotype with a pLAFR1 cosmid clone bank carrying wild type R. meliloti DNA (Charles et al., 1991). Tn5 mutagenesis of the complementing cosmid localized
Fig. 1-2a: Figure adapted from Charles and Finan (1991) showing the genetic linkage map of pRmeSU47b of *R. mellioti* and the two overlapping deletions that delete the *ndvF* (Fix 114) locus.
Fig. 1-2b: Restriction map of pTH21 and pTH38 carrying ndvF and locations of the transposon insertions; From Charles et al. (1991).
the ndvF locus to a 7.3kb BamHI fragment. This fragment was subcloned into pRK7813 and the resulting plasmids pTH38 and pTH39 (two orientations of the insert) were able to restore a Fix' phenotype when transferred into ndvF deleted mutants. Southern blot hybridization revealed that fragments hybridizing to the 7.3kb BamHI fragment were present in at least three other R. meliloti strains, suggesting that ndvF is conserved in R. meliloti (Charles et al., 1991). Subclones of pTH38 as well as the isolation of additional Tn5 and TnphoA insertions localized the ndvF locus to less than 5kb of this DNA. The isolation of active TnphoA fusions, which formed Fix' nodules on alfalfa when recombined on the R. meliloti genome, suggests that the ndvF gene(s) encoded membrane anchored, periplasmic, or secreted protein(s). Each of these fusions were oriented in the same direction suggesting the direction of transcription of the ndvF gene(s) as indicated in Fig. 1-2b (Charles et al., 1991).

In addition to transposon insertions, Ω insertions and deletion mutants of the ndvF locus were created (Charles et al., 1991). Two Ω interposons were cloned in the first and the third EcoRI sites of pTH38 and recombined into a wild type strain to obtain the ndvF mutant strains RmG490 (Rm1021 ndvF-1.7::Ω) and RmG491 (Rm1021 ndvF-5.3::Ω). The RmG439 deletion mutant strain was made by replacing a 12kb HindIII fragment which encompasses the 7.3kb BamHI fragment, with the 3.3kb neomycin-kanamycin (Nm-Km) resistance HindIII fragment of Tn5 and recombining the Nm' marker to the R. meliloti wild type
genome. Both insertion and deletion constructs were confirmed by Southern blot analysis and were Fix when inoculated in alfalfa plants (Charles et al., 1991). Nodule formation was delayed for 2 to 3 days when RmG439 was inoculated on alfalfa plants. The average nodule number per plant inoculated with RmG439 relative to Rm1021 varied. Oresnik et al. (1994) reported that the ndvF mutants exhibit an additional phenotype. When plated on low osmolarity media (GYM), plates on which R. meliloti β-1,2-glucan mutants were not able to grow (Dylan et al., 1990), ndvF mutant strains form colonies with mucoid morphology in contrast to the non-mucoid (dry) phenotype of the wild type strain. This phenotype, specific to ndvF, requires the synthesis of exopolysaccharide II (Esp II) and can be reversed by increasing the osmolarity of the media with the addition of 100mM of various salts. It is interesting that the genes required for exopolysaccharide II synthesis are expressed under phosphate-limiting conditions (Zhan et al., 1991). Increased synthesis of EspII in the ndvF mutants is however not responsible for the symbiotic phenotype of these mutants.

II) The sfx loci

Occasionally some pink Fix+ nodules developed on plants inoculated with the ndvF mutant strains (Oresnik et al., 1994). Rhizobia isolated from these nodules retained the original ndvF mutations but had acquired an extragenic
mutation that fully suppressed the symbiotic deficiency caused by the ndvF mutations. Bacteria from Fix⁺ nodules were isolated from five independent nodulation experiments and designated RmF263 (sfx1), RmF346 (sfx2), RmG203 (sfx4), RmG204 (sfx5) and RmG425 (sfx3) (Oresnik et al., 1994). Upon re-inoculation on alfalfa seedlings, all formed Fix⁺ nodules with plant shoot dry weights similar to that of plants inoculated with the R. meliloti wild type strain. The “suppressor” mutations, however, only partially restored nitrogen fixation of ndvF mutants when inoculated on sweet clover (M. alba cv. Polara) (Oresnik et al., 1994). Also all suppressor mutations suppressed the mucoid phenotype of ndvF mutants when plated on GYM media.

Tn5 (and Tn5 derivatives) insertions which were linked to the suppressor alleles were isolated. These insertions led to the distinction of two different classes of suppressors. Class I, represented by sfx1, sfx4 and sfx5, are flanked by insertions Ω5122::Tn5-132 and Ω5117::Tn5 while class II, represented by sfx2 and sfx3, are not. In addition, only strains RmF346 (sfx2) and RmG425 (sfx3) show sensitivity to the antibiotic bacitracin and the detergents deoxycholate, sodium dodecyl sulfate and sarkosyl. This detergent sensitivity phenotype is only observed in strains lacking a gene designated exoZ. The altered sensitivities of these two strains suggest that sfx2 and sfx3 lead to changes in the permeability of the cell envelope. Tn5 and Tn5-233 insertions linked to sfx2 were isolated employing the deoxycholate sensitivity phenotype to
screen for the presence of the sfx2 mutation. Insertion Ω5258::Tn5 is 70% linked in transduction to the deoxycholate sensitivity of both RmF346 and RmG425, suggesting that sfx2 and sfx3 map to the same locus (Oresnik et al., 1994).

The sfx1 locus was isolated from pink Fix⁺ alfalfa nodules inoculated with RmF114 in which clones from a pRK7813 cosmid library made with partial BamHI digested DNA from RmF263 (sfx1, Δ5408) were mated. This allowed for the isolation of two cosmids (pTH56 and pTH57) that carry a common 18kb BamHI fragment. Both pTH56 and pTH57 suppress the Fix⁺ phenotype when mated back into RmF114 and Rm5408 and direct evidence that pTH56 maps to the sfx1 region was obtained from Southern blot analysis showing that two Tn5-233 insertions linked to sfx1 lay within the pTH56 insert.
D- This Work

The goal of this work was to genetically characterize the two types of suppressor mutations in order to identify the symbiotic function(s) associated with the *ndvF* locus. In the first part of this work, we established that *ndvF* contains four genes *phoCDET* which encode a high affinity phosphate transport system. This was deduced from:

- Sequence homology with the phosphonate transport system in *E. coli* (sequencing of *ndvF* was performed by Shan Dan, MSc. student in the laboratory).
- Inability of *phoCDET* to grow in minimal media containing 2mM Pi as sole phosphorus source.
- Inability of the deleted mutant strain to transport phosphate.

Definite evidence that the symbiotic deficiency of *phoCDET* mutants resulted from the failure of these strains to assimilate Pi came from the genetic characterization of the suppressor mutations.

The *sfx1* mutation is located upstream of an operon in which the deduced protein of one gene shared homology with the low-affinity phosphate transport system (Pit) of *Escherichia coli*. Subsequent analysis revealed that *sfx1* suppression occurs by increasing expression of this locus, which probably results in increased phosphate transport via this alternative transporter.
The sfx2 mutation was mapped to the Pho regulatory genes, encoded by *phoUB*. Evidence presented suggests that while PhoB is a positive regulator of *phoCDET*, it represses the expression of the *pit*-like transporter under phosphate limitation. Suppression of the symbiotic phenotype of *phoCDET* mutants by *sfx2* and *phoUB* mutations appears to occur by derepression of *pit* expression and consequently increased Pi transport via this system.
CHAPTER II

Material and Methods

A- Material

I) Strains, plasmids and transposons

The strains, plasmids and transposons used in this study are listed in table 2-1.

II) Media

All media were sterilized by autoclaving the solutions at 120°C for at least 20 minutes (18 p.s.i.). Solid media were prepared by adding 15g/l Difco Bacto agar to the media prior to being autoclaved. Heat sensitive solutions were filter-sterilized through 0.45μm filters.
# Table 2-1: Strains, plasmids and transposons; Relevant characteristics; Source, reference or construction.

<table>
<thead>
<tr>
<th>Strains, plasmids and transposons</th>
<th>Relevant characteristics</th>
<th>Source, reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhizobium meliloti</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SU47</td>
<td>wild type (strain RCR2011)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>Rm1021</td>
<td>SU47 str-21</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>Rm5000</td>
<td>SU47 rif-5</td>
<td>Finan et al. (1988)</td>
</tr>
</tbody>
</table>

**Transposon insertion banks**

| Bank MM1                          | ca. 6000 Tn5 insertions in Rm1021 background (=Rm5354) | Laboratory collection |
| Bank GS2                          | ca. 2000 Tn5-233 insertions in Rm5000 background     | Laboratory collection |
| Bank OT1                          | ca. 14000 Tn5-132 insertions in Rm1021 background (=Rm5353) | Laboratory collection |

**Rm1021 derivatives**

<p>| Rm5439                            | pck-1::TnV                   | Finan et al. (1988)               |
| Rm5408                            | ΔΩ5033-5007::Tn5-233; Fix'    | Charles and Finan (1991)          |
| RmF114                            | ΔΩ5033-5064::Tn5-233; Fix'    | Charles and Finan (1991)          |
| RmF222                            | (=Rm8002) Pho'               | Long et al. (1988)                |
| RmF263                            | ΔΩ5033-5007::Tn5-233, sfx1; Fix' | Oresnik et al. (1994)        |
| RmF288                            | ntrAΩ76::Tn5                 | O. Yaroch                      |
| RmF346                            | ΔΩ5033-5064::Tn5-233, sfx2; Fix' | Oresnik et al. (1994)        |
| RmG203                            | ΔΩ5033-5007::Tn5-233, sfx4; Fix' | Oresnik et al. (1994)        |
| RmG212                            | Lac'                        | J. Glazebrook                  |
| RmG204                            | ΔΩ5033-5064::Tn5-233, sfx5; Fix' | Oresnik et al. (1994)        |
| RmG425                            | ΔΩ5033-5064::Tn5-233, sfx3; Fix' | Oresnik et al. (1994)        |
| RmG439                            | ΔΩ5033-5064::Tn5-233, sfx3; Fix' | Oresnik et al. (1994)        |
| RmG439                            | ΔG439 (ΔndvF HindIII::Nm (12kb)) = ndvFΔG439; Fix' | Charles et al. (1991)          |
| RmG490                            | ndvF-1.7Ω Sp' = phoCΩ490; Fix' | Charles et al. (1991)          |
| RmG490                            | ndvF-5.3Ω Sp' = phoCQ491; Fix' | Charles et al. (1991)          |
| RmG479                            | Ω5025::Tn5, sfx2             | Oresnik et al. (1994)          |</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>Map Position</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RmG497</td>
<td>Ω5033::Tn5-233, sfx2</td>
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</tr>
<tr>
<td>RmG514</td>
<td>ndvΔAG439, sfx2, Ω5025::Tn5; Fix*</td>
<td>Oresnik et al. (1994)</td>
</tr>
<tr>
<td>RmG549</td>
<td>ΔΩ5033-5064::Tn5-233, Q5256:: Tn5</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG551</td>
<td>ΔΩ5033-5064::Tn5-233, Ω5258:: Tn5</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG552</td>
<td>ΔΩ5033-5064::Tn5-233, Ω5259:: Tn5</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG591</td>
<td>sfx1</td>
<td>Oresnik et al. (1994)</td>
</tr>
<tr>
<td>RmG639</td>
<td>Ω5262::Tn5-233, sfx2</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG640</td>
<td>Ω5263::Tn5-233, sfx2</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG641</td>
<td>Ω5264::Tn5-233, sfx2</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG702</td>
<td>sfx1-orfAΩ10A::Tn5</td>
<td>I. Oresnik</td>
</tr>
<tr>
<td>RmG762</td>
<td>phoCΩ490, sfx1; Fix*</td>
<td>pTH56Ω10A::Tn5→(pPH1J1)→G591, Nm'</td>
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<tr>
<td>RmG763</td>
<td>phoTΩ491, sfx1; Fix*</td>
<td>ΦRmG490 → RmG591, Sp'</td>
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<td>RmG764</td>
<td>sfx1Ω2-2::Tn5</td>
<td>ΦRmG491 → RmG591, Sp'</td>
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<td>RmG765</td>
<td>sfx1-orfAΩ2-3::Tn5</td>
<td>pTH90Ω2-2::Tn5→(pPH1J1)→G591, Nm'</td>
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<td>RmG766</td>
<td>sfx1-pitΩ2-5::Tn5</td>
<td>pTH90Ω2-3::Tn5→(pPH1J1)→G591, Nm'</td>
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<tr>
<td>RmG771</td>
<td>sfx1-pitΩ3-3::Tn5</td>
<td>pTH90Ω2-5::Tn5→(pPH1J1)→G591, Nm'</td>
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<tr>
<td>RmG774</td>
<td>sfx1-pitΩ3-10::Tn5</td>
<td>pTH90Ω3-3::Tn5→(pPH1J1)→G591, Nm'</td>
</tr>
<tr>
<td>RmG777</td>
<td>sfx1-pitΩ3-16::Tn5</td>
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<tr>
<td>RmG781</td>
<td>sfx1ΩE::Tn5</td>
<td>pTH90Ω3-16::Tn5→(pPH1J1)→G591, Nm'</td>
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<td>RmG783</td>
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<td>pTH90ΩJ::Tn5→(pPH1J1)→G591, Nm'</td>
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<td>RmG821</td>
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<td>ΦRmG765 → RmG762, Nm'</td>
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<td>RmG823</td>
<td>phoCΩ490, sfx1-pitΩ2-5::Tn5; Fix*</td>
<td>ΦRmG766 → RmG762, Nm'</td>
</tr>
<tr>
<td>RmG827</td>
<td>phoCΩ490, sfx1-pitΩ3-3::Tn5; Fix*</td>
<td>ΦRmG771 → RmG762, Nm'</td>
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<tr>
<td>RmG830</td>
<td>phoCΩ490, sfx1-pitΩ2-10::Tn5; Fix*</td>
<td>ΦRmG774 → RmG762, Nm'</td>
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<tr>
<td>RmG833</td>
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<td>ΦRmG777 → RmG762, Nm'</td>
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<tr>
<td>RmG837</td>
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<td>ΦRmG783 → RmG762, Nm'</td>
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<tr>
<td>RmG838</td>
<td>phoCΩ490, sfx1-orfAΩ10A::Tn5; Fix*</td>
<td>ΦRmG792 → RmG762, Nm'</td>
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<td>RmH138</td>
<td>ndvΔAG439, sfx1; Fix*</td>
<td>ΦRmG439 → RmG591, Nm'</td>
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<td>RmH318</td>
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<td>ΦRmG551 → RmG497, Nm' Gm'-Sp'</td>
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<tr>
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<td>phoCΩ490, sfx2; Fix*</td>
<td>ΦRmG490 → RmG479, Sp' Nm'</td>
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<td>RmH392</td>
<td>Lac+, phoEΩ219::Tn5B20</td>
<td>pTH21Ω19::Tn5B20→(pPH1J1)→G212, Nm'</td>
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<tr>
<td>RmH393</td>
<td>Lac+, Ω17::Tn5B20</td>
<td>pTH21Ω17::Tn5B20→(pPH1J1)→G212, Nm'</td>
</tr>
<tr>
<td>RmH394</td>
<td>Lac+, phoDΩ7A::Tn5B20</td>
<td>pTH21Ω7A::Tn5B20→(pPH1J1)→G212, Nm'</td>
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</table>
Lac', \(\textit{pho}T_\text{O}4B::\text{Tn5B20}\)
RmH396
\(\textit{O}\phi\text{ho}12::\text{Tn5-132}, \text{white on XPhos}\)
RmH397
\(\textit{O}\phi\text{ho}12::\text{TnV}\)
RmH399
\(\textit{O}\phi\text{ho}U10::\text{TnV}\)
RmH405
\(\textit{O}\phi\text{ho}27::\text{Tn5, 100\% linked to RmH396}\)
RmH406
\(\textit{O}\phi\text{ho}B3::\text{Tn5-132, white on X-Phos, 66\% linked to }\Omega5258::\text{Tn5}\)
RmH407
\(\textit{O}\phi\text{ho}B8::\text{Tn5-132, white on X-Phos, 64\% linked to }\Omega5258::\text{Tn5}\)
RmH428
\(\textit{O}\phi\text{ho}B8::\text{TnV}\)
RmH429
\(\textit{O}\phi\text{ho}U10::\text{Tn5-132, white on X-Phos, 60\% linked to }\Omega5258::\text{Tn5}\)
RmH430
\(\textit{O}\phi\text{ho}B3::\text{TnV}\)
RmH541
Lac', \(\textit{O}\phi\text{ho}28::\text{Tn5B20, 100\% linked to RmH396}\)
RmH610
\(\textit{O}\phi\text{ho}27::\text{TnV}\)
RmH615
Lac', \(\textit{O}\phi\text{ho}B3::\text{TnV}\)
RmH616
Lac', \(\textit{O}\phi\text{ho}B8::\text{TnV}\)
RmH617
Lac', \(\textit{O}\phi\text{ho}U10::\text{TnV}\)
RmH623
\(\textit{O}\phi\text{ho}U10::\text{TnV, pho}C\Omega490; \text{Fix}^+\)
RmH624
\(\textit{O}\phi\text{ho}B8::\text{TnV, pho}C\Omega490; \text{Fix}^+\)
RmH625
\(\textit{O}\phi\text{ho}B3::\text{TnV, pho}C\Omega490; \text{Fix}^+\)
RmH627
\(\text{sfx1}\Omega12A::\text{TnphoA}\)
RmH635
Lac', \(\text{sfx1-or}fA\Omega2-3::\text{Tn}\)
RmH636
Lac', \(\text{sfx1-pit}Q3-10::\text{Tn5}\)
RmH659
Lac', \(\text{ndv}\Delta\text{G439}\)
RmH661
Lac', \(\text{pho}T\Omega491\)
RmH662
Lac', \text{wild type promoter }\text{pitQ}::\text{lacZmobSp, ort I}\)
RmH667
Lac', \(\text{pho}C\Omega490\)
RmH668
Lac', \text{wild type promoter }\text{pitQ}::\text{lacZmobSp, ort II}\)
RmH689
\(\text{ndv}\Delta\text{G439, sfx1 promoter }\text{pitQ}::\text{lacZmobSp, ort II}\)
RmH692
Lac', \(\Omega5258::\text{Tn5},\)
RmH693
Lac', \(\Omega5258::\text{Tn5, sfx2}\)
RmH695
Lac', \(\text{sfx1 promoter }\text{pitQ}::\text{lacZmobSp, ort II}\)
RmH753
\(\text{ndv}\Delta\text{G439, sfx1 promoter }\text{pitQ}::\text{lacZmobSp, ort I}\)
RmH754
Lac', \text{wild type promoter }\text{pitQ}::\text{lacZmobSp, ort I}, \(\text{O}\phi\text{ho}U10::\text{TnV}\)
RmH755
Lac', \text{wild type promoter }\text{pitQ}::\text{lacZmobSp, ort I}, \(\text{O}\phi\text{ho}B8::\text{TnV}\)
RmH756
Lac', \text{wild type promoter }\text{pitQ}::\text{lacZmobSp, ort I}, \(\text{O}\phi\text{ho}B3::\text{TnV}\)

\(\text{pTH21}\Omega4B::\text{Tn5B20} \rightarrow (\text{pPH1J1}) \rightarrow \text{G212, Nm}'\)
This work
\(\text{TnV replacement of RmH396, O}'\ Nm'\)
\(\text{TnV replacement of RmH429, O}'\ Nm'\)
This work
This work
This work
\(\text{TnV replacement of RmH407, O}'\ Nm'\)
This work
\(\text{TnV replacement of RmH406, O}'\ Nm'\)
isolated by 3V03 student
\(\Phi\text{RmH609} \rightarrow \text{Rm1021, Nm}'\)
\(\Phi\text{RmH430} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmH428} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmH429} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmH429} \rightarrow \text{RmG490, Sp}' \text{Nm}'\)
\(\Phi\text{RmH428} \rightarrow \text{RmG490, Sp}' \text{Nm}'\)
\(\Phi\text{RmH430} \rightarrow \text{RmG490, Sp}' \text{Nm}'\)
\(\text{pTH276O12A: TnphoA} \rightarrow (\text{pPH1J1}) \rightarrow \text{G591, Nm}'\)
\(\Phi\text{RmG822} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmG830} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmG439} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmG491} \rightarrow \text{RmG212, Sp}'\)
\(\Phi\text{RmH657} \rightarrow \text{RmG212, Sp}' \text{Tc}'^2\)
\(\Phi\text{RmG490} \rightarrow \text{RmG212, Sp}'\)
\(\Phi\text{RmH658} \rightarrow \text{RmG212, Sp}' \text{Tc}'^2\)
\(\text{pTH352 recombined onto RmH138, Nm}' \text{Sp}' \text{Tc}'^2\)
\(\Phi\text{RmH138} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmH138} \rightarrow \text{RmG212, Nm}'\)
\(\Phi\text{RmH689} \rightarrow \text{RmG212, Sp}' \text{Tc}'^2\)
\(\text{pTH351 recombined onto RmH138, Nm}' \text{Sp}' \text{Tc}'^2\)
\(\Phi\text{RmH399} \rightarrow \text{RmH662, Nm}' \text{Sp}' \text{Tc}'^2\)
\(\Phi\text{RmH428} \rightarrow \text{RmH662, Nm}' \text{Sp}' \text{Tc}'^2\)
\(\Phi\text{RmH430} \rightarrow \text{RmH662, Nm}' \text{Sp}' \text{Tc}'^2\)
RmH757  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl II; ΩphoU10::TnV
RmH758  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl II; ΩphoB8::TnV
RmH759  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl II; ΩphoB3::TnV
RmH765  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl I; Δ5258::Tn5, sfx2
RmH766  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl I; Ω5258::Tn5
RmH768  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl II; Ω5258::Tn5
RmH769  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl I; ndvFΔG439
RmH770  Lac⁺, wild type promoter pitΩ::lacZmobSp, orl II; ndvFΔG439
RmH771  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I
RmH785  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; ndvFΔG439
RmH791  Lac⁺, phoCΩ490; ΩphoU10::TnV
RmH792  Lac⁺, phoCΩ490; ΩphoB8::TnV
RmH793  Lac⁺, phoCΩ490; ΩphoB3::TnV
RmH794  Lac⁺, phoCΩ490; Ω5258::Tn5, sfx2
RmH795  Lac⁺, phoCΩ490; Ω5258::Tn5
RmH796  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl II, ΩphoU10::TnV
RmH797  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl II, ΩphoB8::TnV
RmH798  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl II, ΩphoB3::TnV
RmH807  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl II, ndvFΔG439
RmH808  Lac⁺, ntrAΩ76::Tn5
RmH836  ΩphoU10::TnV
RmH837  ΩphoB8::TnV
RmH838  ΩphoB3::TnV
RmH842  phoCΩ490, sfx1Ω12A::TnphoA; Fix'
RmH850  ΩphoU10::Tn5-233
RmH851  ΩphoB8::Tn5-233
RmH852  ΩphoB3::Tn5-233
RmH859  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; Ω5258::Tn5
RmH860  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; Ω5258::Tn5, sfx2
RmH861  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; ΩphoU10::TnV
RmH862  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; ΩphoB8::TnV
RmH863  Lac⁺, sfx1 promoter pitΩ::lacZmobSp, orl I; ΩphoB3::TnV

ΦRmH399 → RmH668, Nm' Sp' Tc²
ΦRmH428 → RmH668, Nm' Sp' Tc²
ΦRmH430 → RmH668, Nm' Sp' Tc²
ΦRmH318 → RmH662, Nm' Sp' Tc²
ΦRmH318 → RmH662, Nm' Sp' Tc²
ΦRmH318 → RmH668, Nm' Sp' Tc²
ΦRmH318 → RmH668, Nm' Sp' Tc²
ΦRmH430 → RmH662, Nm' Sp' Tc²
ΦRmH430 → RmH662, Nm' Sp' Tc²
ΦRmH753 → RmG212, Sp' Tc²
ΦRmH439 → RmH771, Nm' Sp' Tc²
ΦRmH399 → RmH667, Nm' Sp'
ΦRmH428 → RmH667, Nm' Sp'
ΦRmH430 → RmH667, Nm' Sp'
ΦRmH318 → RmH667, Nm' Sp'
ΦRmH318 → RmH667, Nm' Sp'
ΦRmH399 → RmH695, Nm' Sp' Tc²
ΦRmH428 → RmH695, Nm' Sp' Tc²
ΦRmH430 → RmH695, Nm' Sp' Tc²
ΦRmH439 → RmH695, Nm' Sp' Tc²
ΦRmF288 → Rm1021, Nm'
ΦRmH399 → Rm1021, Nm'
ΦRmH428 → Rm1021, Nm'
ΦRmH430 → Rm1021, Nm'
ΦRmH627 → RmG762, Nm' Sp'
ΦRmH847 → Rm1021, Gm' Sp'
ΦRmH848 → Rm1021, Gm' Sp'
ΦRmH849 → Rm1021, Gm' Sp'
ΦRmH318 → RmH771, Nm' Sp' Tc²
ΦRmH318 → RmH771, Nm' Sp' Tc²
ΦRmH399 → RmH771, Nm' Sp' Tc²
ΦRmH428 → RmH771, Nm' Sp' Tc²
ΦRmH430 → RmH771, Nm' Sp' Tc²
**Rm5000 derivatives**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Markers and Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RmH590</td>
<td>( \Omega phoU10::TnV )</td>
</tr>
<tr>
<td>RmH591</td>
<td>( \Omega phoB8::TnV )</td>
</tr>
<tr>
<td>RmH592</td>
<td>( \Omega phoB3::TnV )</td>
</tr>
<tr>
<td>RmH593</td>
<td>( \Omega pho27::Tn5 )</td>
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<tr>
<td>RmH598</td>
<td>( \Omega pho27::Tn5-233 )</td>
</tr>
<tr>
<td>RmH609</td>
<td>( \Omega pho27::TnV )</td>
</tr>
<tr>
<td>RmH657</td>
<td>wild type promoter ( pit\Omega::lacZmobSp, ortl )</td>
</tr>
<tr>
<td>RmH658</td>
<td>wild type promoter ( pit\Omega::lacZmobSp, ortll )</td>
</tr>
<tr>
<td>RmH847</td>
<td>( \Omega phoU10::Tn5-233 )</td>
</tr>
<tr>
<td>RmH848</td>
<td>( \Omega phoB8::Tn5-233 )</td>
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<tr>
<td>RmH847</td>
<td>( \Omega phoB3::Tn5-233 )</td>
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**Escherichia coli**

<table>
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<th>Strain</th>
<th>Markers and Notes</th>
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<tr>
<td>MM294A</td>
<td>pro82 thi1 hsdR17 supE44 endA1</td>
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<tr>
<td>MT607</td>
<td>MM294A recA56</td>
</tr>
<tr>
<td>MT609</td>
<td>thy36 polA1 Sp'</td>
</tr>
<tr>
<td>MT614</td>
<td>MT607 ( \Omega::Tn5, Nm-Km' )</td>
</tr>
<tr>
<td>MT616</td>
<td>MT607 (pRK600)</td>
</tr>
<tr>
<td>MT621</td>
<td>MM294A malf::TnphoA, Nm-Km'</td>
</tr>
<tr>
<td>G312</td>
<td>MT607 ( \Omega5::Tn5B20, Nm-Km' )</td>
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<tr>
<td>HB101</td>
<td>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsK20 xyl-5 mtl-1 ( \text{r} \text{m} )</td>
</tr>
<tr>
<td>DH5α</td>
<td>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 ( \Delta(argF-lacZYA) U169 )</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>supE44 hsdR17 endA1 gyrA46 relA1 thi recA ( [\text{F' proAB lac}\text{R} \text{Z}\text{ΔM15 Tn10 (Tc')} ) )</td>
</tr>
</tbody>
</table>
**Plasmids**

pUC18,19  
Cloning vector, ColE1 oriV, Ap′

pUC118, 119  
pUC18/19 containing intergenic (IG) region of M13

pRK2013  
ColE1 replicon with RK2 transfer region, Nm-Km′

pRK600  
pRK2013 npt::Tn9; Cm′, Nm-Km′

pRK602  
pRK600::Tn5, Cm′, Nm-Km′

pRK607  
pRK2013::Tn5-233; Nm-Km′ Gm′ Sp′

pRK7813  
RK2 derivative carrying pUC9 polylinker and cos site, Tc′

pLAFRI  
IncP cosmid cloning vector, Tc′

pGS220  
Tn5 in deletion derivative of pBR322, Ap′ Nm-Km′

pPH1JI  
IncP, Gm′ Sp′ Cm′

pMP220  
IncP, promoterless lacZ, Tc′

pTF1  
pBR322::TnV, Ap′, Nm-Km′

pLMS  
pUC18 containing the lacZ mob Sp′ cassette

pTH21  
pLAFR1 clone carrying Rm1021 ndVF

pTH22  
pLAFR1 clone carrying Rm1021 ndVF

pTH38  
7.3kb BamHI subclone of pTH22 in pRK7813, carries ndVF

pTH56  
pRK7813 clone carrying sfx1

pTH57  
pRK7813 clone carrying sfx1

pTH61  
12kb HindIII fragment of pTH56 in pUC19, carrying sfx1

pTH90  
12kb HindIII fragment of pTH61 in pRK7813, carrying sfx1

pTH191  
2.1kb EcoRI fragment of pTH90 in pUC118; orfI; orfA pit region

pTH192  
2.1kb EcoRI fragment of pTH90 in pUC118; orfII. orfA pit region

pTH270  
pRK600 O5A::Tn5B20; Nm-Km′

pTH276  
4.8kb HindIII/Sacl fragment of pTH90 in pRK7813, carrying orfA pit

pTH282  
pLAFR1 cosmid clone restoring AP of RmH399; # 3

pTH284  
pLAFR1 cosmid clone restoring AP of RmH399; # 7

pTH286  
pLAFR1 cosmid clone restoring AP of RmH399; # 11

pTH287  
SalI ligation of RmH430 (phoB3::TnV)

pTH292  
SalI ligation of RmH399 (phoU10::TnV)

pTH304  
2.6kb HindIII/Smal fragment of pTH90 in pRK7813, orfA pit region

pTH305  
2.7kb HindIII/EcoRV fragment of pTH90 in pRK7813, carrying orfA pit

pTH306  
0.5kb EcoRI fragment of pTH90 in pUC118, orfI, end of pit

Yanisch-Perron et al. (1985)

Vieira and Messing (1987)

Figurski and Helinski (1979)

Finan et al. (1986)

Finan et al. (1985)

De Vos et al. (1986)

Jones and Guterson (1987)

Friedman et al. (1982)

De Vos et al. (1986)

Beringer et al. (1978)

Spainik et al. (1987)

Furuichi et al. (1985)

M. Hynes


Oresnik et al (1994)

Oresnik et al (1994)

I. Oresnik

T.C. Charles

This work

This work

B. Driscoll

This work

B. Schoeman

B. Schoeman

B. Schoeman

B. Schoeman

B. Schoeman

This work

This work

This work

This work
pTH310 2.6kb partial (2.1kb + 0.5kb) EcoRI fragment of pTH90 in pUC118, carrying orfA pit
pTH311 Sall ligation of RmH428 (phoB8::TnV)
This work
pTH343 pTH306 in which the SacII-PstI fragment as been substituted by a 30 mer oligo (for construction of the chromosomal lacZ fusion)
B. Schoeman
pTH344 PstI/EcoRI fragment of pTH343 subcloned in pBR322
This work
pTH347 2.6kb partial EcoRI fragment of pTH90 in pRK7813 (ort I), carrying orfA pit
This work
pTH348 2.6kb partial EcoRI fragment of pTH90 in pRK7813 (ort II), carrying orfA pit
This work
pTH351 pTH344 containing the lacZmobSp cassette of pUC18LMS subcloned as a SmaI fragment (ort I)
This work
pTH352 pTH344 containing the lacZmobSp cassette of pUC18LMS subcloned as a SmaI fragment (ort II)
This work
pTH354 4.8kb HindIII/SacI fragment isolated from wild type genomic DNA and containing the wild type orfA pit allele.
This work
pTH365 2.1kb EcoRI fragment of pTH90 in pMP220 (ort I); sfx1 promoter pit-lacZ fusion
This work
pTH367 pTH365 Δ SphI; sfx1 promoter orfB-lacZ fusion
This work
pTH376 2.1kb EcoRI fragment of pTH354 in pMP220 (ort I); wild type promoter pit-lacZ fusion
This work
pTH378 pTH376 Δ SphI; wild type promoter orfB-lacZ fusion
This work
pTH380 4.8kb HindIII/SacI fragment of pTH90 in pUC118; carrying orfA pit
This work
pTH391 2.6kb partial EcoRI fragment of pTH354 in pRK7813 (ort II) carrying wild type orfA pit locus
This work
pTH396 0.9kb HindIII-XhoI fragment of pTH191 in HindIII-SalI sites of pBR322, orfA promoter region
This work
pTH397 0.6kb HindIII-EcoRV fragment of pTH191 in HindIII-EcoRV sites of pBR322, orfA promoter region
This work
### Plasmids and phage

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13K07</td>
<td>M13 derivative, helper phage</td>
<td>Vieira and Messing (1987)</td>
</tr>
<tr>
<td>ΦM12</td>
<td><em>R. meliloti</em> bacteriophage</td>
<td>Finan et al. (1984)</td>
</tr>
</tbody>
</table>

### Transposons

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn5</td>
<td>Nm'·Km'· Sm'</td>
<td>Berg and Berg (1987)</td>
</tr>
<tr>
<td>Tn5-132</td>
<td>Ot'</td>
<td>Berg and Berg (1987)</td>
</tr>
<tr>
<td>Tn5-233</td>
<td>Gm', Sp'</td>
<td>De Vos et al. (1986)</td>
</tr>
<tr>
<td>TnV</td>
<td>Tn5 containing pSC101 oriV, Nm'·Km'</td>
<td>Furuichi et al. (1985)</td>
</tr>
<tr>
<td>TnPhoA</td>
<td>Tn5 derivative generating AP translational fusion, Nm'·Km'</td>
<td>Manoil and Beckwith (1985)</td>
</tr>
<tr>
<td>Tn5B20</td>
<td>Tn5 derivative generating lacZ transcriptional fusion, Nm'·Km'</td>
<td>Simon et al. (1989)</td>
</tr>
</tbody>
</table>

Abbreviations are as followed: Ap, ampicillin; AP, alkaline phosphatase; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Ot, oxytetracycline; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Rif, rifampicin, Lac, lactose utilization genes; mob, mobilization region of RK2; ort I and II, orientation I and II; oriT, origin of transfer; oriV, origin of vegetative replication; X-Phos, 5-bromo-4-chloro-3-indolyl phosphate. ΦM12 transducing lysates are indicated by Φ followed by the strain number. For strain construction, an arrow indicates transduction from the indicated ΦM12 transducing lysates to the recipient strain. Homogenotizations followed by transduction of the insertion recombined back into the recipient strain are indicated by an arrow and parentheses enclosing the introduced incompatible plasmid pH1JI.
• LB (Luria-Bertani) was prepared by dissolving 10g of tryptone, 5g of yeast extract and 5g sodium chloride per liter (l) of solution. 4ml/l sodium hydroxide (1M) was added to the broth while 1ml/l of sodium hydroxide and 15g/l of agar was added for the preparation of plates. 2.5mM of magnesium sulfate and calcium chloride were added to medium used for *Rhizobium* growth (LBmc).

• M9 minimal medium was prepared by dissolving 5.8g disodium hydrogen phosphate, 3g potassium dihydrogen phosphate, 0.5g sodium chloride and 1g of ammonium chloride per liter of solution (1x M9). The solution was autoclaved without agar or other supplement to prevent precipitation. To prepare M9 plates, sterile 2x M9 medium was mixed with an equal volume of sterile distilled water containing 30g/l agar. Filter sterilized solutions of magnesium sulfate, calcium chloride, biotin and D-glucose (or other carbon sources) were added to the autoclaved M9 media at final concentrations of 1mM, 0.25mM, 3μg/ml and 15mM, respectively.

For transduction experiments, ½ LB-½ 1x M9 agar plates were used. This media was prepared by mixing 150ml of the LB solution containing 30g/l agar with 150ml of 1x M9 solution.
• GYM media was made of 1mM sodium glutamate, 2.5mM mannitol, 1mM dipotassium hydrogen phosphate pH 6.8, 0.5mM magnesium sulfate, 0.5mM calcium chloride and 0.2% (w/v) yeast extract. GYM/NaCl was prepared by adding 100mM sodium chloride to the GYM media.

• MOPS-buffered minimal media was made of 40mM morpholinopropionate sulfonic acid/20mM potassium hydroxide pH 7.4, 20mM ammonium chloride, 2mM magnesium sulfate, 1.2mM calcium chloride, 100mM sodium chloride, 3μg/ml biotin, 5ml/l of a partially purified factor isolated from yeast extract (provided by Dr. Watson) and 15mM glucose (or succinate) as carbon source. When required a phosphorus source was added at a final concentration of 2mM.

Note: The MOPS/KOH solution, the biotin and the carbon source were filter-sterilized through a 0.45μm filter and added after the rest of the ingredients were autoclaved. The glassware used was phosphate-free (rinsed in HNO₃ 6M solution).

• Water containing 15g/l agar was used to prepare plates for the germination of plant seedlings.
III) Antibiotics and other additives

For the preparation of plates, antibiotics were added to the media at the following concentrations (in $\mu$g/ml): Neomycin (Nm) 200, Spectinomycin (Sp) 200, Gentamicin (Gm) 20, Oxytetracycline (Ot) 0.5, Kanamycin (Km) 20, Rifampicin (Rif) 20, Chloramphenicol (Cm) 10, Ampicillin (Ap) 100, Tetracycline (Tc) 10 and Streptomycin (Sm) 200. All antibiotics were dissolved in water (and filter sterilized through a 0.45$\mu$m filter) except for Ot and Tc that were dissolved in 95% ethanol, Cm dissolved in 50% ethanol and Rif dissolved in dimethylsulfoxide.

For liquid media or media containing more than one antibiotic, each antibiotic was used at half the concentration.

Other additives include (final concentration in $\mu$g/ml): thymidine 60, IPTG (isopropyl $\beta$-D-thiogalacto-pyranoside) 30, X-Gal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside) 50 and X-Phos (5-bromo-4-chloro-3-indolyl phosphate) 60. The phosphorus sources used for the growth experiments in the MOPS-buffered minimal media included ethylphosphonate (EP; Aldrich), aminoethylphosphonate (AEP; Sigma), methylphosphonate (MP; Aldrich), aminomethylphosphonate (AMP; Aldrich), L-$\alpha$-glycerophosphate (G3P; Sigma), glucose-6-phosphate (Glc6P; Sigma) and O-phospho-L-serine (PSeR; Sigma).
B- Methods

1) Growth conditions

*Rhizobium* and *E. coli* strains were grown at 30°C and 37°C, respectively. Liquid cultures inoculated with a single colony were shaken to provide aeration.

For growth in the MOPS-buffered minimal media the following procedure was used: The cells were grown for 24 hours in LBmc, spun down, washed once with phosphate-free MOPS media (MOPS P0), and 5μl of cells resuspended in MOPS P0 was used to inoculate 5ml of MOPS media (1/1000 dilution). For the growth experiments over time, an additional step was added in order to reduce the polyphosphate content of the cells. This otherwise allowed significant growth in MOPS P0 media. The washed cells were inoculated in 5ml of MOPS P0 media (starting absorbency at OD$_{600}$ was 0.05 approximately) and grown for 24 hours. The optical density of the cultures were diluted to an OD$_{600}$ of 0.2 and 5μl was used to inoculate 5ml of MOPS media containing various phosphorus sources.
II) Genetic modification of a cell

1- Preparation of competent cells and transformation

This method was used to transfer plasmid DNA into competent *E. coli* cells. The cells were made competent using a calcium chloride solution as follows:

0.1ml of an overnight saturated culture was used to inoculate 100ml of LB broth. The cells were grown to an OD$_{600}$ of 0.2-0.3 and centrifuged for 5 min at 4000rpm, 4°C. The subsequent steps were performed on ice. The pellet of cells was resuspended in 50ml of an ice cold solution of 50mM calcium chloride and 20mM potassium acetate (pH 6.2) and left on ice for one hour. After 10 min centrifugation at 4000rpm, 4°C the cells were resuspended in 10ml of the above solution containing 20% glycerol. 0.5ml aliquots were stored at -70°C.

For the transformation, 100μl of competent cells were thawed on ice then mixed with 1μg of plasmid DNA. The mixture was left on ice for an hour, heat shocked for 2 min at 37°C and put back on ice for 1 min. 1ml of LB was added to the cells and the cultures were grown for one hour before being pelleted, resuspended in about 200μl LB and plated on two LB plates containing antibiotic to select for the plasmid. Colonies appeared after 12 hours growth at 37°C.
2- Conjugal mating

The transfer of a foreign plasmid DNA into *R. meliloti* cell required mating between the plasmid donor strain (*E. coli*) and the recipient cell. Mating of mobilisable plasmids was facilitated by the presence of an *E. coli* strain, MT616, containing the “helper” plasmid pRK600 which supplied the RK2 transfer genes in trans. This procedure is referred to as triparental mating.

The strains involved in the mating were grown to late log phase in LB containing appropriate antibiotics for the *E. coli* strains (donor and helper) and in LBmc for the *Rhizobium* recipient strain. The *E. coli* cultures were spun down and resuspended in LB media to eliminate of the antibiotics. 20\(\mu\)l of each culture were mixed and the resulting 60\(\mu\)l was spotted on an LB plate. As controls, 20\(\mu\)l of each strain culture was spotted on their own on an LB plate. After 16 hours incubation at 30\(^\circ\)C, the spots were resuspended in 1ml saline (0.85% sodium chloride) and 100\(\mu\)l of the suitable dilution was plated on LB containing antibiotics selecting for the plasmid mated and the recipient strain. Colonies appeared after 3-4 days incubation at 30\(^\circ\)C.

3- Homogenotization

Insertions within genes cloned in the plasmid pRK7813 (IncP) were recombined onto the chromosome via homologous recombination. To select for homogenotes, the pRK7813 plasmid was cured by mating into the *Rhizobium*
strain a second plasmid (pPH1JI) of the same incompatibility group (IncP) and selecting for the second plasmid, the selective marker of the recombined insert, and the recipient cell. The procedure was as follows: An overnight culture of a purified *Rhizobium* transconjugant colony grown in LBmc Nm (50μg/ml) (to select for a Tn5 insert, for example) was mixed with an equal volume (1ml) of a log phase culture of the *E. coli* J53 strain grown in LB Gm (10μg/ml) (Note: J53 contains pPH1JI a self-transmissible plasmid). The cells were pelleted, resuspended in 1ml LB and 100μl was spotted on an LB plate. After 16 hours incubation at 30°C the spot was resuspended in 1ml saline and 100μl was plated at 10^0 and 10^{-1} dilutions on LB Sm (100μg/ml) Nm (100μg/ml) and Gm (70μg/ml). Colonies appeared 3 to 5 days after incubation at 30°C. The colonies were purified 3 times by plating on the above selective media and the loss of the pRK7813 plasmid was confirmed by the sensitivity of single colonies on LB Tc (10μg/ml) plates. To eliminate of the incompatible plasmid (pPH1JI), the recombined insertion was transduced back into the recipient strain and the structure of the recombinants was checked by Southern blot (see below).

4- Generalized Transduction and Phage preparation

Generalized transduction involved the transfer of genomic DNA fragment from one strain to another via a transducing phage that is able to pack random
fragments of host DNA. The phage used for transduction in *R. meliloti* is ΦM12 (Finan et al., 1984).

a) To **prepare a ΦM12 phage lysate**, 5ml of an overnight culture grown in LBmc (the phage requires calcium for infection) was diluted to an OD$_{675}$ of 0.4-0.5 in the same media. 100µl of Rm1021 lysate was added to the culture which was incubated on a 30°C rotating wheel for 12 hours to allow complete lysis of the cells. 200µl of chloroform was added to the lysate to kill any remaining viable cells. The lysate was vortexed briefly and the cell debris was allowed to settle at 4°C for at least one hour. The top 4ml of the lysate was transferred to a new tube and centrifuged for 5 min, at 4000rpm, 4°C. The lysate should give a titer of $10^{10}$-$10^{11}$ PFU/ml (PFU = plaque forming units) and can be stored at 4°C for several years.

b) To **determine the phage titer**, the lysate was diluted to $10^{-10}$ in LBmc. 0.1ml of the $10^{-8}$, $10^{-9}$ and $10^{-10}$ dilutions was mixed with 0.1ml of overnight culture of Rm1021 grown in LBmc. Following incubation at room temperature for 15 min, 3ml of LBmc containing 0.5% agar (cooled to 50°C) was added. The mixture was poured onto an LBmc plate and after solidification of the soft agar, the plates were incubated overnight at 30°C. The PFU per ml was calculated by multiplying the number of plaques obtained by the dilution factor and dividing by 0.1.
c) **Transduction of genetic markers** from one strain to another was performed by mixing 1ml of 1/30 diluted lysate, made from the strain containing the marker to be transduced, with 1ml of the recipient culture (OD₆₇₅ ~ 1). This gave a multiplicity of infection of ~ 0.5. The mixture was left on the bench for 20-25 min to allow adsorption. 2.5ml of saline was added and the cells were pelleted. The supernatant was removed and the cells were washed once with 2.5ml saline. The pellet of cells was resuspended in 1ml saline. 0.1ml was plated on ½ LB-½ 1x M9 agar plates supplemented with the antibiotic(s) selecting for the transduced marker. As a control, the diluted phage lysate and the recipient cells were plated on this selective media as well.

**d) Linkage between two markers**

To determine whether two markers (A and B) were linked (closely located), marker A was transduced into a strain containing a chromosomal insertion of marker B. The transductants, isolated by selecting for marker A, were checked for the presence of marker B by patching 50 to 100 colonies on media selecting for marker B. The percentage of transductants that have lost their resistance for marker B provided the % linkage between the two markers. The physical distance between two markers can be determined from the Wu equation (Wu, 1966): \( c = (1-d/L)^3 \), where c represents the cotransduction frequency, d the distance between markers (kb) and L the length of DNA in the transducing particle (160kb for ΦM12; Charles and Finan, 1990; Finan et al., 1984).
5- **Tn5, Tn5-B20 and TnphoA mutagenesis**

Transposon Tn5 is 5.7kb in size and carries genes encoding resistance for Nm-Km, bleomycin and Sm which are flanked by inverted repeats (IS50 elements). They can transpose from one replicon to another at a frequency of about $10^{-4}$ to $10^{-6}$ per recipient. Two techniques were used to create Tn5 mutations:

a) **Random mutagenesis**

Random mutagenesis of *R. meliloti* genomic DNA was performed by introducing a suicide plasmid (plasmid that cannot replicate in *Rhizobium*) carrying Tn5 or derivatives (such as pRK602 for Tn5; pTH270 for Tn5-B20) from an *E. coli* strain and selecting for the Nm\(^r\) recombinants.

b) **Directed mutagenesis**

In this case, Tn5 insertions into cloned DNA fragment were isolated from an *E. coli* strain carrying a transposon on its chromosome (i.e. MT614 for Tn5; G312 for Tn5-B20; MT621 for TnphoA). The cloned plasmid DNA was then transferred, by triparental mating, into another *E. coli* strain (MT609; Sp\(^r\) polA) by selecting for the plasmid (Tc\(^r\)) carrying the transposon (Km\(^r\))

c) **Transposon replacements**

The replacement of Tn5-132 (Ot\(^r\)) and Tn5-233 (Gm\(^r\)-Sp\(^r\)) insertions with TnV was performed by homologous recombination between the flanking IS50 elements. Plasmid pTF1 from *E. coli* was mated to the *R. meliloti* (Sm\(^r\)) strains
carrying the insertions to be replaced. As pTF1 cannot replicate in *Rhizobium*, recombinants were selected as Sm⁻¹-Nm⁻¹ strains and true replacements were obtained after screening for the loss of Ot or Gm-Sp resistance markers. To replace a Tn5 insertion with Tn5-233, the Tn5 marker was first transduced into the Rif⁻ strain Rm5000. The replacement was then performed by mating pRK607 into this strain, selecting for Rif⁻ Gm⁻¹-Sp⁻ transconjugants and screening for the loss of the Nm marker.

III) DNA and RNA preparation

1- DNA preparation

a) Alkaline lysis

Mini- and large-scale plasmid DNA preparations (preps) were performed as described in Sambrook et al. (1989; second edition, vol. 1, pp. 1.38-39). For mini-preps (1.5ml of cells), 100μl of Solution I (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA pH 8), 200μl Solution II (0.2N NaOH, 1% SDS) and 150μl Solution III (60ml 5M K-acetate, 11.5ml glacial acetic acid, 28.5ml H₂O) were used. Large scale preps were further purified using the LiCl; PEG/NaCl precipitation procedure described in Sambrook et al. (1989; pp. 1.41).
b) *Single-stranded DNA*

*E. coli* strains containing either pUC118 or pUC119 cloned DNA were grown in LB Ap^{50} to an OD_{600} of 0.1. 5\mu l of M13K07 helper phage (~ 10^{11} phage/ml) was added to the culture which was subsequently grown at 30°C for at least 8 hours. 1.5ml of culture was centrifuged at room temperature (RT) for 5 min (12,000rpm). The supernatant was transferred to a new tube, centrifuged a second time and transferred to a tube containing 250\mu l of PEG/NaCl (polyethylene glycol 6000/2.5M sodium chloride). The tubes were vortexed, left 10 min at RT to allow precipitation and centrifuged 10 min at 12,000rpm. The pellet was dissolved in 200\mu l TES (20mM Tris-HCl pH 7.4, 0.1mM EDTA, 10mM NaCl) and the phage proteins were extracted with phenol:chloroform (3:1; v:v) as follows: the mixture was vortexed for 1 min, left on ice for 5 min, vortexed again and centrifuged at RT for 5 min (12,000rpm). This extraction was repeated once followed by a chloroform extraction. The ssDNA was precipitated by adding 0.1 volume (vol) of 3M NH\textsubscript{4}Ac and 2.5 vol of ethanol. After centrifugation the pellet was washed with ethanol 70% and dissolved in 20\mu l of T_{10}E_{1}. 5\mu l was checked on an agarose gel.

c) *R. meliloti* genomic DNA

5ml LBmc overnight cultures were centrifuged at RT for 5 min (5,000rpm). The pellets were washed once with 0.85% NaCl (saline), once with TES (10mM Tris-HCl pH 8, 25mM EDTA, 150mM NaCl) and were resuspended in 2.5ml of
$T_{10}E_{25}$ to which 250$\mu$l lysozyme (2mg/ml in $T_{10}E_{25}$) was added. The mixture was incubated 15 min at 37°C. 300$\mu$l of sarkosyl-protease solution (5mg/ml protease E (Sigma) was dissolved in $T_{10}E_{25}$ and incubated at 37°C for 2 hours. After this autolysis period, 10% sarkosyl was added. The solution was kept at -20°C) was then added and the incubation at 37°C was continued for another hour. The mixture was phenol extracted by adding 1.5ml of phenol, mixing gently by inversion, and centrifuging 20 min at 5,000rpm (RT). The aqueous (upper) layer was withdrawn very carefully avoiding interface and was re-extracted once with phenol and once with chloroform. The DNA was precipitated by adding 1 vol of isopropanol and mixing by inversion. The precipitated DNA was picked up with a glass pipette, washed once with 70% ethanol, once with 95% ethanol, air dried and dissolved in 300$\mu$l $T_{10}E_1$. 1$\mu$l of 100$\mu$g/ml RNAase was added to the dissolved DNA and the mixture was incubated for 30 min at 37°C. After phenol:chloroform and chloroform extractions, the DNA was precipitated by adding 0.1 vol of 3M NH$_4$Ac and 2 vol of ethanol. The precipitated DNA was picked up with a glass pipette, washed with 70% ethanol, then 95% ethanol, air dried for a 2 min and dissolved in 200$\mu$l $T_{10}E_1$. 5$\mu$l was checked on an agarose gel.
2- *R. meliloti* RNA preparation (using hot phenol)

When working with RNA, gloves were worn at all times and all the solutions were treated with diethylpyrocarbonate (DEPC) 0.1% (carcinogen) as follows: the DEPC was mixed well in the solutions; left overnight at room temperature and autoclaved.

To prepare RNA from *R. meliloti*, 100μl of saturated LBmc culture was used to inoculate 200 ml of LBmc. When an OD₆₀₀ between 0.3-0.8 was reached, the cells were centrifuged for 5 min at 5,000rpm (RT), resuspended in 50 ml of TES (10mM Tris-HCl pH 7.5, 1mM EDTA, 100mM NaCl), centrifuged again as above and resuspended in 6ml of TES. 1.5 ml were dispensed into four eppendorf tubes that were kept on ice at all time from this point on. The tubes were centrifuged for 5 min at 12,000rpm (4°C) and the pellets were dissolved in 500μl of ice cold Extraction Buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, 5% sucrose, 300mM CH₃COONa, 1% SDS and 1% β-mercaptoethanol). 300μl of hot phenol (90°C) were added to each tube. The tubes were mixed gently by inversion and 300μl of chloroform:iso-amyl-alcohol (24:1) were added. After vortexing the mixture, the tubes were centrifuged 10 min at 12,000rpm (4°C). The aqueous (upper) phase was transferred to a new tube and 2.5 vol of 95% ethanol was added. After precipitation, the pellets were dissolved in 50μl H₂O (treated with DEPC 0.1%). Two rounds of precipitation with 150μl of 4M NaAc, pH 7 (treated with DEPC) were performed. Both times, the RNA was allow to
precipitate for at least 16 hours at -20°C. After the second precipitation, the RNA was dissolved in 100μl H₂O (treated with DEPC 0.1%) and the remaining proteins were extracted once with phenol:chloroform (1:1) and once with chloroform. The RNA was precipitated by adding 1/10 volume of 3M NaAc pH 5 and 250μl 95% EtOH and centrifuged for 10 min at 12,000rpm (4°C). The pellet was dissolved in 100μl H₂O (DEPC treated). 250μl of 95% ethanol was added to the RNA which was stored at -20°C. When needed, the RNA was precipitated by adding 10μl of 3M NaAc pH 5 and after centrifugation, the RNA was dissolved in 100μl H₂O (DEPC treated). 5μl was checked on an 1.2% agarose gel.

IV) Southern blot and colony hybridization

1- Southern blot

Digested DNA fragments separated on an agarose gel were transferred to a nylon filter as follows: the gel, stained with ethidium bromide, was photographed (with a ruler beside the molecular weight markers) and the DNA was depurinated for 10 min in 0.25M HCl solution, denatured in 1.5M NaCl, 0.5M NaOH solution for 2x 15 min and neutralized in 1M Tris-HCl pH 7.4, 1.5M NaCl, solution for 2x 15 min. The gel was rinsed with water after each solution. The denatured DNA was transferred to nylon membrane by capillary as described in
Sambrook et al. (1989; pp. 9-34 and 9-38,40) using 5x SSC for the transfer (20x SSC pH 7: 175.3g/l NaCl; 88.5g/l trisodium citrate).

2- Colony hybridization

Nylon membranes, cut to match the size of a petri-dish, were placed on the colonies and left there for three min (to increase the transfer, the membrane can be pressed gently with a glass bar). The membrane and the petri-dish were both marked to be able to orient the membrane after hybridization. The membranes (colony side face up) were then transferred in petri-dishes containing whatman paper soaked with the following solutions; i) 0.2M NaCl, 1% SDS for 5 min; ii) 1.5M NaCl, 0.5M NaOH for 10 min; iii) 1M Tris-HCl pH 7.4, 1.5M NaCl, for 15 min and iv) 2x SSC for 5 min. The DNA was fixed to the membrane by UV irradiation (2 min). The membranes were then soaked in 2x SSC, 0.1% SDS for 5 min and the cell debris were gently removed with a Kimwipe.

3- Preparation of the Digoxigenin-probe

The DNA fragment used to make the probe was isolated from an agarose gel using the GeneClean II kit from Bio101 (cat # 001-400) and Digoxigenin (Dig)-labeled following the protocol of Boehringer Mannheim (Kit # 1093 657). About 10ng of DNA was denatured (10 min in boiling water followed by 3 min in an
ice/ethanol bath) and added to the following labeling mix from the kit: 4μl of hexanucleotide mix (random primer for DNA synthesis), 4μl dNTP mix (1mmol/l dATP, 1mmol/l dCTP, 1mmol/l dGTP, 0.65mmol/l dTTP, 0.35mmol/l digoxigenin-11-dUTP), 2μl (4U) Klenow DNA polymerase and water to a volume of 40μl. The reaction mixture was placed in a 37°C water bath overnight. The reaction was stopped by adding 2μl of 0.5M EDTA pH 8. The DNA-probe was precipitated by adding 5μl of 2.5M NaCl and 150μl ethanol and left at least 30 min at -70°C prior to centrifugation. The pellet was washed once with 70% ethanol, once with 95% ethanol, air dried and dissolved in 50μl T_20 E_1.

4- Estimation of the yield of the Dig-labeled DNA probe

This was performed by comparing the labeled probe with dilutions of labeled control DNA of known concentration. The labeled DNA control (concentration of 5ng/μl) was diluted 5; 50; 500; 5000; 50000 times to give final concentrations of 1ng; 100pg; 10pg; 1pg; 0.1pg per μl. 1μl of each dilution was spotted onto a nylon membrane. 1μl of the undiluted probe and 1μl of the probe diluted 10; 100; 1000; 10000 times were also spotted on the nylon membrane beneath the control spots. The DNA was fixed to the membrane by UV irradiation (2 min) and the labeled DNA was detected as follows: The following steps were done under agitation and at room temperature. The membrane, wetted in Buffer 1 (100mM Tris-HCl pH 7.5, 150mM NaCl), was incubated i) in Buffer 2 (same as
Buffer 1 but containing 0.5% blocking reagent) for 15 min and ii) 15 min in the
diluted Dig-antibody Solution (1:5000 in Buffer 1), which was washed off with
Buffer 1 (2x 15 min). The membrane was then equilibrated at pH 9.5 by
incubating it 2 min in Buffer 3 (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM
MgCl₂) and after pouring off Buffer 3, 10ml of Color Substrate Solution (10ml
Buffer 3 containing 45μl NBT and 35μl X-Phos) was added to the membrane.
The color was allowed to develop in the dark at 37°C, without agitation. The NBT
solution (nitroblue tetrazolium salt) was at a concentration of 75mg/ml in 70%
dimethylformamide. The X-Phos solution (5-bromo-4-chloro-3-indolyl phosphate
sodium salt) was dissolved in water at a concentration of 50mg/ml. The reaction
was stopped by putting the membrane in water for 5 min, then allowed to air dry.
The spot intensities of the sample were compared to the control to estimate the
concentration of the probe.

5- Hybridization

The membrane was placed in a plastic box with 50ml prehybridization
solution (5x SSC, 0.1% sarkosyl, 0.02% SDS, 5% blocking reagent and 50%
deionized formamide) for 1 hour at 42°C. The prehybridization solution was
replaced with 20ml of hybridization solution, prehybridization solution containing
the denatured probe (see "preparation of the Dig-probe" for the denaturation of
the probe), and incubated for 24 hours at 42°C. The hybridization solution was
poured off (and stored at -20°C for future use) and the membrane was washed 2x 5 min in wash 1 (2x SSC, 0.1% SDS) at room temperature and 2x 15 min in wash 2 (0.1x SSC, 0.1% SDS) at 42°C. The membrane was air dried or used immediately for detection.

6- Detection

The detection was performed as described in “Estimation of the yield of the Dig-labeled probe” (# 4) with the following modifications; the membrane was rehydrated in Buffer 1, incubated in Buffer 2 for 30 min and another 90 min in the diluted Dig-antibody Solution. The subsequent steps were identical to those described in “Estimation of the yield of the Dig-labeled probe”.

7- Stripping of the probe

The membrane was not dried if it was to be stripped and reprobed. To remove the color precipitate the membrane was incubated in a glass plate containing dimethylformamide heated on a heater block to 50-60°C (No flame, dimethylformamide is flammable!!!; work in a fume hood). The dimethylformamide solution was changed several times until the blue color was removed. The membrane was then rinsed thoroughly in water. The probe was removed, by incubating the membrane 2x 10 min in the Alkaline Probe-Stripping
Solution (0.4M NaOH, 0.1% SDS) at 37°C and rinsing it in 2x SSC solution. The filters were reprobed by starting at the prehybridization step.

V) Plant growth

Plant growth experiments were performed in a nitrogen-free environment to determine the ability of mutant strains to nodulate and fix nitrogen. The plants were grown in Leonard jars containing Jensen's media.

1- Preparation of the Jars

Leonard assemblies consist of a plastic jar sitting inside a 250ml beaker with a cotton wick extending from a hole at the bottom of the jar. The jar was filled with a nitrogen-free sand/vermiculite (1:1) mixture and 250ml of 1x Jensen's media (1g/l CaHPO₄, 0.2g/l K₂HPO₄, 0.2g/l MgSO₄.7H₂O, 0.2g/l NaCl, 0.1g/l FeCl₃, and 1ml/l trace minerals. The trace minerals solution was composed of 1g/l H₃BO₃, 1g/l ZnSO₄.7H₂O, 0.5g/l CuSO₄.5H₂O, 0.5g/l MnCl₂.4H₂O, 1g/l NaMoO₄.2H₂O, 10g/l EDTA, 2g/l NaFe-EDTA and 0.4g/l Biotin). The whole apparatus was autoclaved for 2 hours.
2- Sterilization of the seeds

Alfalfa seeds (*Medicago sativa* cultivar Iroquois; 100 seeds weighing approximately 0.25g) were surface sterilized 5 min in 95% ethanol followed by 20 min in a 50% bleach solution (sodium hypochlorite). The seeds were then rinsed with sterile water at least 8 times over an hour and were spread on water agar plates (15g/l agar) using a flamed spatula. The plates were placed in the dark at room temperature until germination (2-3 days).

3- Inoculation of the seedlings

Ten germinated seedlings were transferred to an autoclaved Leonard jar assembly and put in the growth chamber (day = 18 hours, 21°C; night = 6 hours, 18°C). After 2 days, 0.1ml of a *R. meliloti* saturated cultures (OD$_{600}$ 1 to 1.5) diluted in 10 ml of sterile water ($1\times10^7$-$1\times10^8$ cells) were used to inoculate a Leonard jar assembly containing emerging seedlings. Three jars were inoculated with the same bacterial strain (total of 30 seedlings). The plants were grown up to one month and were watered as needed with sterile distilled water.

4- Acetylene reduction assay

To determine the nitrogenase activity of the nodules, an acetylene reduction assay (ARA) was performed. The roots of three plants from each jar were inserted in a 30 ml bottle and sealed with a serum stopper. 3ml of
acetylene gas were injected in each bottle and the quantity of acetylene reduced (ethylene produced) after 15 min was monitored by injecting an 0.2ml sample into a Hewlett-Packard 5890 gas chromatograph (GC; air 34psi, H₂ 12psi, N₂ 65psi). The ethylene peaks were integrated using the HP3365 Series II Chemstation computer program and the nmol of acetylene reduced per hour per plant was determined as follows:

Using the formula \( V = nRT/Pr \) and the fact that at standard temperature (T) and pressure (Pr), 1 mole of gas has a volume of 22.4l (= R), 1ml of gas then contains \( 1/2.24 \times 10^4 \) moles. 1ml of 506ppm ethylene \((506 \text{ part}/10^6 = 5.06 \times 10^{-4})\) will have \( 5.06 \times 10^{-4}/2.24 \times 10^4 = 2.26 \times 10^{-8} \) moles and 0.2ml of 506ppm ethylene, \( 2.26 \times 10^{-8}/5 = 4.52 \) nmol. By relating the unit peak area, when 0.2ml of a 506ppm ethylene standard was injected to the GC, to the number of mole, the number of mole per unit area can be determined \((Z_{\text{nmol/unit}})\). The acetylene reduction per plant per hour of the sample can then be calculated from the general formula \((\text{ARA} =)\):

\[
\text{# area units of sample} \times Z_{\text{nmol/unit}} \times 150 \ (0.2\text{ml is 1/150 the bottle volume})
\]

3 plants. 0.25 hours (= 15 min)
5- Determination of the plant dry weight

The plant shoots from each jar were cut and dried in a paper bag for one week in a 100°C oven. The dry weight (in mg per plant) was determined by weighting the dried plants of each bag and dividing by the number of shoots.

VI) Uptake experiments

1- Phosphate and succinate transport assays

Phosphate and succinate uptake experiments were done as described in Bardin et al. (1996) and Yarosh et al. (1989; see also Chapter III of this thesis). The inhibition experiments were performed by adding the inhibitors to the assay mixture 15 s prior to adding the $^{33}$P-labeled phosphate.

2- Protein determination

Protein concentration was determined by the Bradford method using the Biorad protein assay dye (Coomassie blue; Bradford, 1976). The cells were solubilized by diluting them in 1N NaOH (1:1 ratio) and boiling them for 15 min. The protein concentration of a sample was determined from a bovine serum albumin (BSA) standard curve made using the microassay procedure (concentration from 0 to 10μg protein per ml of 0.5M NaOH). The procedure was as follows: 100μl of 0 to 100μg/ml BSA (or protein sample) in 0.5M NaOH were
added to 700µl water and 200µl of Biorad solution (undiluted). The solutions were vortexed, incubated 5 min at RT and the OD_{595} was determined.

3- **Phosphate determination**

The phosphate concentration of a solution was determined using the method of Martin and Tolbert (1983). The reagent (made fresh each time) was prepared by mixing 10 ml of water with 3ml of concentrated sulfuric acid (H₂SO₄) in which 0.25g of ammonium molybdate ((NH₄)₆ MoO₂₄) was dissolved. The volume of the solution was then brought to 50ml with water and 1g of ascorbic acid was added (yellow solution; if the solution turned blue this indicated that the water was contaminated with phosphate). A standard curve was prepared by mixing 500µl of the reagent with 500µl of a potassium phosphate solution with concentration ranging from 0 to 50µM. The mixtures were vortexed and incubated at 37°C for 90 min and the OD_{820} was measured. The phosphate concentration of samples was determined by mixing 500µl sample with 500µl reagent and comparing the optical densities obtained from the samples to those of the standard curve.
VII) **Enzymatic assays**

1- **β-Galactosidase assay**

0.5ml of overnight grown cells (for which OD\textsubscript{600} was measured) was mixed with 0.5ml of Buffer Z (pH 7; 60mM Na\textsubscript{2}HPO\textsubscript{4}.7H\textsubscript{2}O, 40mM NaH\textsubscript{2}PO\textsubscript{4}.H\textsubscript{2}O, 10mM KCl anhydrous, 1mM MgSO\textsubscript{4}.7H\textsubscript{2}O and 2.7ml/l of 2-mercaptoethanol added just before use); 20μl chloroform and 10μl SDS 0.1%. The tubes were vortexed and equilibrated at 30°C for 5 min. The reaction was started by adding 0.2ml of 4mg/ml O-nitrophenyl-β-D-galactoside (ONPG) dissolved in Buffer Z (at 37°C). When the solution turned yellow, the reaction was stopped by adding 0.5ml of 1M Na\textsubscript{2}CO\textsubscript{3}. The tubes were centrifuged 5 min at 12,000rpm and the optical density at 420nm (OD\textsubscript{420}) was measured.

The β-Galactosidase activity, in Miller units was calculated using the following formula:

\[(1000 \cdot \text{OD}_{420}) / (\text{OD}_{600} \cdot \Delta T \cdot V)\]

with \(\Delta T\): reaction time (min) and \(V\): initial volume of culture used (ml).

2- **Alkaline phosphatase assay**

The alkaline phosphate (AP) activity was determined after the cells (to be assayed) were centrifuged and resuspended in a 1M Tris-HCl pH 8 buffer to an OD\textsubscript{600} of approximately 0.1. The OD\textsubscript{600} was precisely recorded for each tube. 3ml of the diluted cells were equilibrated at 30°C for 5 min and the reaction was started by adding 500μl of 4mg/ml nitrophenyl phosphate (NPP). After
development of a yellow coloration (or after 1 hour) the reaction was stopped by adding 600μl of 1M phosphate solution. The tubes were centrifuged 5 min at 12,000rpm and the OD₄₂₀ was measured. The alkaline phosphatase activity was calculated using the following formula:

\[(1000 \cdot \text{OD}_{420}) / (\text{OD}_{600} \cdot \Delta T)\]

with ΔT: reaction time (min)

VIII) DNA Sequencing (see also chapter IV)

DNA sequencing was performed using single-stranded DNA, following the dideoxy chain termination method according to the protocol of United States Biochemicals for the Sequenase 2.0 enzyme and using \[\alpha^{-35}\text{S}]dATP. 7μl of single stranded DNA (about 1μg) was mixed with 1μl of primer (0.5pmol/μl) and 2μl of 5x Sequenase buffer. The mixture was incubated 2 min at 65°C and the tubes were allowed to cool down slowly to room temperature to allow annealing. Labeling of the reactions was performed by adding 1μl DTT, 2μl labeling mix (diluted 5x), 0.5μl \[\alpha^{-35}\text{S}]dATP (1000Ci/mmol) and 2μl Sequenase enzyme (diluted 8x) to the annealing mixture. The labeling mixture was incubated for 3 min at RT. The reactions were terminated by adding 3.5μl of the labeling mix to 2.5μl of each of the four dideoxy nucleotide Termination Mix (the ddA Termination Mix was a 50mM NaCl solution containing 80μM of each dGTP,
dCTP, dATP and dTTP in addition to 8µM ddATP) and incubating the reactions 5 min at 37°C. The reactions were stopped by adding 4µl of Stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF) and stored at -20°C for up to one week. The sequencing reactions (2µl) were run on a 6% denaturing polyacrylamide gel (acryl (19):bisacryl (1); 7M urea) after denaturing the samples by incubating them 2 min at 80°C followed by 1 min on ice. The gel was run at 2000 volts.
CHAPTER III

A phosphate transport system is required

for symbiotic nitrogen fixation in *Rhizobium meliloti*

A- Introduction

This chapter presents the genetic characterization of the symbiotic locus, *ndvF*, identified on the second megaplasmid (pRmSU47b) of *Rhizobium meliloti* 1021 (Charles and Finan, 1991; Charles et al., 1991). The locus was located in a 5kb region within a 7.3kb *BamHI* fragment. Isolation of active Tn*phoA* fusions to the locus indicated that *ndvF* encodes membrane anchored, periplasmic or secreted protein(s). Light microscopy of nodules elicited by *ndvF* mutant strains showed very few infected cortical cells, suggesting that nodule development is blocked early in the infection process (Charles et al., 1991).

The results reported here show that *ndvF* contains four genes (*phoCDE*/*T*) that together encode for an ABC-type transporter involved in the uptake of phosphate and possibly phosphonate (organophosphorus compounds with a direct C-P bond). The results are presented in the following publication:

My contribution to the paper includes:

- Growth experiments in MOPS-buffered minimal media containing phosphate, phosphorus and phosphonate compounds.
- Phosphate and succinate uptake experiments.
- Participation in the primer extension experiments.

This chapter is completed with experiments investigating the affinity and specificity of the PhoCDET transporter for phosphate.

B- Results

1- *A phosphate transport system is required for symbiotic nitrogen fixation by Rhizobium meliloti.*

(see publication)
A Phosphate Transport System Is Required for Symbiotic Nitrogen Fixation by *Rhizobium meliloti*

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The bacterium *Rhizobium meliloti* forms *N*₂-fixing root nodules on alfalfa plants. The *ndvF* locus, located on the 1,700-kb *pEXO* megaplasmid of *R. meliloti*, is required for nodule invasion and *N*₂ fixation. Here we report that *ndvF* contains four genes, *phoCDET*, which encode an ABC-type transport system for the uptake of *P*₂ into the bacteria. The *PhoC* and *PhoD* proteins are homologous to the *Escherichia coli* phosphonate transport proteins *PhnC* and *PhnD*. The *PhoT* and *PhoE* proteins are homologous to each other and to the *E. coli* phosphonate transport protein *PhnE*. We show that the *R. meliloti* *phoD* and *phoE* genes are induced in response to phosphate starvation and that the *phoC* promoter contains two elements which are similar in sequence to the *PHO* boxes present in *E. coli* phosphate-regulated promoters. The *R. meliloti ndvF* mutants grow poorly at a phosphate concentration of 2 mM, and we hypothesize that their symbiotic phenotype results from their failure to grow during the nodule infection process. Presumably, the *PhoCDET* transport system is employed by the bacteria in the soil environment, where the concentration of available phosphate is normally 0.1 to 1 μM.

In the biogeochemical nitrogen cycle, much of the reduction of atmospheric *N*₂ to ammonia occurs in bacteria within plant root nodules. Molecular genetic studies of bacterial symbiotic mutants have resulted in the identification of nodulation (*nod*) and nitrogen fixation (*nif*) genes whose products are directly involved in the biochemical events which give rise to these nodules. The products of the bacterial *nod* genes synthesize lipooligosaccharide molecules which trigger the dedifferentiation of the plant root cortical cells destined to develop into the nodule primordia (30, 49). Many of the *nif* genes are involved in the regulation or synthesis of the *N*₂-fixing enzyme nitrogenase and its accessory proteins (17). The *nod* genes are induced in response to plant flavonoid signals (40), while in planta expression of the nitrogen fixation genes appears to be controlled by oxygen concentration (12, 48).

Mutants which are classified as defective in nodule development (*ndv*) have also been identified. In the alfalfa symbiont *Rhizobium meliloti*, *ndvA, ndvB, ndvF*, and *exo* mutants form "empty nodules" which contain very few infected cells and fail to fix *N*₂ (Fix') (15, 19, 29). While the *ndvA* and *ndvB* gene products are involved in production of cyclic β-(1,2)-glucans and *exo* mutants lack a sugioligocen exopolysaccharide, the precise role(s) of these polysaccharides in nodule development remains unclear (14, 28). In *exo* mutants, the symbiotic phenotype appears to result from an inability of these mutants to grow in planta during the nodule infection process (41).

*R. meliloti* contains two megaplasmids, *pSYM* and *pEXO*, which are 1,600 and 1,700 kb, respectively (7, 24, 25, 42). The *nod* and *nif* genes are located on *pSYM* and we have previously reported the identification and cloning of the *ndvF* locus, which is located on the *pEXO* megaplasmid. Here we report results showing that *ndvF* contains four genes (*phoCDET*) which encode an ABC-type (periplasmic binding protein-dependent) transport system which transports phosphate (*P*₂), and likely alkylphosphonates, across the cytoplasmic membrane of *R. meliloti*. We hypothesize that the symbiotic phenotype of the *ndvF* mutants is a direct result of their failure to obtain sufficient phosphorus for growth during the infection process.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, and media. Bacteria were grown on LB or LMM medium with antibiotic concentrations as previously described (7). MOPS-minimal medium contained 40 mM morpholinopropanesulfonic acid (MOPS), 3 mM KOH, 30 mM NaCl, 2 mM MgSO₄, 100 mM NaCl, 1.2 mM CaCl₂, and 0.3 μg of thymine per ml. Glucose or succinate was added as a carbon source in a final concentration of 15 mM. *R. meliloti* strain 1021 is a streptomycin-resistant derivative of SUT7. Strain RM G439 is a strain RM 1021 derivative in which the 12-kb *HindIII* fragment containing the *ndvF* locus was replaced with the internal neomycin resistance (*Nm*') *HindIII* fragment of TTn5; R. *meliloti* RM G439 (pRMR901) and Rm G439 (phoF1) are strain RM 1021 derivatives in which an *NpsI* fragment was inserted in the first and third EcoRI sites within *ndvF*, respectively (8). pTH38 contains the complete *ndvF* locus cloned in an 7.3-kb BamHI fragment in pRK713, and pTH38 is a pLAFR1 cosmID clone carrying the complete *ndvF* locus within a 22-kb region (8).

**Genetic techniques, DNA manipulations, and sequencing**. Bacterial mutations were performed as previously described (7). TTn5-B20 (45) insertions in *ndvF* were identified following mutagenesis of TTn5 by using *Escherichia coli* MT607::TTn5-B20 as transposon donor. TTn5-B20 insertions in the plasmid DNA were determined by restriction and DNA sequence analysis. Standard methods were used for plasmid DNA isolation, restriction digestion, agarose and polyacrylamide gel electrophoresis, ligations, and transformation (44).

Plasmids pTH38 and pTH38HB: TTn5 were used as the sources of material for DNA sequencing (Fig. 1). Restriction fragments were subcloned into pUC18 and pUC119 and used for construction of unidirectional nested deletions by exonuclease III treatment followed by S1 nuclease digestion (21). The nucleotide sequence was determined from single-stranded DNA obtained from host strain XL2Blue (Stratagene) following infection with helper phage M13K07 (52). DNA sequencing was performed by dideoxy chain termination according to the protocol of United States Biochemicals for the Sequenase 2.0 enzyme, by using [α-³²P]dATP (NEN/DuPont) and 7-deaza dGTP (Pharmacia). Both strands of DNA were sequenced. TaqPho insertion sites in pTH38 were sequenced directly from double-stranded DNA by using the *phoA*-specific primer (5'-AAATCGCC CCTGAGC-3'). TTn5-B20 insertion sites were sequenced by using the universal -20 (222) primer (5'-GTAAAACGACGGCCAGT-3'). 7A, 19, 4B, and 17 had inserted at nucleotide positions 1792, 2862, 4841, and 5211, respectively (Fig. 1).
DNA and derived protein sequences were analyzed with the PC Gene (IntelliGenetics) Blast 2, CLUSTAL (23), and Top Pred II (10) software packages.

RNA extraction and primer extension. RNA was extracted from wild-type R. meliloti 1021 cultures as previously described (39). The purity and quality of the RNA were checked by electrophoresis through a 1.2% agarose gel with Tris-acetate-EDTA (TAE) running buffer. The contaminating DNA was removed by treatment with 2× U of RNase-free DNase (Boehringer-Mannheim) per ml for 30 min at 37°C in the presence of 6 U of RNase inhibitor (RnaGuard; Pharmacia) per ml. This was followed by a phenol-chloroform-isooamyl alcohol (25:24:1) extraction and ethanol precipitation of the RNA.

To identify the transcription start site of the phoC mRNA, a specific oligonucleotide (5'GGCTTTTCCGGCAGATGCGGCAGTTCGTCTGCA) complementary to the beginning of the coding region of the gene was synthesized (Mobsx, McMaster University). End labelling and extension of the primer with avian myeloblastosis virus reverse transcriptase were performed as previously described (39).

Transport and growth experiments. For phosphate transport assays, LBmC-grown cells were washed twice with MOPS-minimal medium, subcultured (1:20 dilution) into phosphate-free MOPS-minimal medium, and incubated with shaking (200 rpm) overnight at 30°C. Cells were harvested by centrifugation, washed at 4°C in MOPS minimal medium (without P [−P]), resuspended in the same medium to an optical density at 600 nm of 5, and stored at 4°C. For phosphate uptake assays, the cells were diluted 1:20 into MOPS minimal medium (+P) and incubated at 30°C for 5 min. A 12-μl volume of [32P]orthophosphate (60 μCi/μl) was added to a final concentration of 10 μM. Aliquots (100 μl) were removed at various times, placed on a 0.45-μm pore-size nitrocellulose filter (pore size 1 μM, HPO), and immediately washed with MOPS minimal medium (−P). Filters were dried, placed in liquid scintillation vials with scintillation fluid, and counted in a scintillation spectrophotometer. For all experiments, we used chloroform-treated cells to determine the amount of background binding of [32P]P to the bacterial cells. Saturate uptake experiments employing [32P]orthophosphate (2.5 μCi/μl) at a final concentration of 40 μM were done as previously described (56).

To test strains for P utilization ability, cultures grown in LBmC were diluted 1:1000 into 5 ml of MOPS-buffered minimal medium containing the indicated phosphorus sources at 2 mM. Cultures were grown with shaking at 30°C and growth was monitored by measuring the optical density at 600 nm.

β-Galactosidase and alkaline phosphatase (AP) assays were performed with aliquots of cells from 5-ml cultures as previously described (39, 56).

Nucleotide sequence accession number. The nucleotide sequence (5.705 bp) of the ndvF locus of R. meliloti has been deposited in GenBank under accession number U59229.

RESULTS

Nucleotide sequence of the ndvF locus. The ndvF locus was previously localized to a 7.3-kb BamHI fragment cloned in plasmid pTH38. Transposon Tn5 insertion mutagenesis of pTH38 followed by complementation analysis further localized ndvF to a 5-kb region between the Fix− insertions B8 and B9 (Fig. 1). We have determined the nucleotide sequence for both strands of the 5.705-bp region from insertion B8 to the EcoRI site located 493 nucleotides beyond insertion B9 (Fig. 1). Analysis of this sequence revealed four nonoverlapping open reading frames (ORFs) encoding proteins of 270, 300, 320, and 505 amino acids, which we designated PhoC, PhoD, PhoE, and PhoT, respectively (Fig. 1). The four genes are transcribed in the direction predicted in a previous study employing AP gene fusions to the ndvF locus (8). A clear G−C bias was observed at the third nucleotide position of the codons within each ORF (79 to 83°C), relative to the general G−C content (64°C), and the results (not shown) of a codon preference plot employing an R. meliloti codon usage table suggested that the four predicted genes were expressed in R. meliloti (20). Putative ribosome-binding sites were identified for all ORFs. Between 5 and 9 bp upstream of the ATG start codons (AGGAAN,ATG, AGGAAN,ATG, CGGAAN,ATG, and TAAAGN,ATG, respectively), the intergenic regions were 117 bp long between phoC and phoD, 172 bp long between phoD and phoE, and 9 bp between phoE and phoT. While it appears likely that these four genes compose an operon, further experiments are required to conclusively establish this supposition.

Characterization of the encoded proteins. A BLASTx search of GenBank revealed that the ndvF-encoded proteins were similar to the E. coli phnC, phnD, and phnE gene products (2, 9). The PhoC and PhoD proteins were homologous to PhnC and PhnD, respectively, while the PhoT and PhoE proteins were homologous to each other and to the E. coli PhnE protein. The E. coli phn genes are required for the transport and catabolism of phosphonates and are transcribed as an 11-kb operon (54). Phosphonates are organophosphorus compounds which have direct C-P bonds rather than the common C-O-P phosphodiester linkage. Sequence analysis and experimental evidence suggest that the E. coli phnC, phnD, and phnE genes encode a phosphonate and phosphate transport system of the ABC (ATP-binding cassette) class (54). In gram-negative bacteria, these transport systems are made up of a periplasmic binding protein, one or two integral membrane proteins, and a hydrophilic ATP-binding protein (13, 22).
CLUSTALV alignments of the *R. meliloti* Pho and *E. coli* Phn proteins are shown in Fig. 2. The PhoC and PhnC proteins are 43% identical. They are homologous to the highly conserved ATPase component of the ABC transport systems and contain the two Walker motifs associated with many nucleotide-binding proteins (residues 54 to 42 and 165 to 170) (Fig. 2, boxes A and B). The amino terminus of the PhoD protein has characteristics of an export signal sequence, such as a basic amino terminus, a hydrophobic core, and in this case two cleavage sites (amino acids 19–20 and 21–22) which conform to the (−3, −1) rule (37). This is in agreement with the periplasmic location of the substrate-binding protein present in all ABC transporters that mediate solute uptake (22). The *R. meliloti* PhoT protein contains 505 amino acid residues, and while it is much larger than the *R. meliloti* PhoE (320 amino acids) and *E. coli* PhnE (276 amino acids) proteins, its C-terminal 200 amino acids have a high level of homology with those two proteins (Fig. 2). This size difference is reminiscent of that found between the analogous integral membrane proteins MalF (514 amino acids) and MalG (296 amino acids) of the ABC-type maltose transport system of *E. coli* (11, 18).

**Membrane topology of the PhoCDET proteins.** In a previous study, we isolated mutants with ndfA::TphoA gene fusions which expressed AP activity (8). Such fusions are generally only active when AP is fused to an exported protein or to the external domain of a transmembrane protein (35). Analysis of the DNA sequence of the ndfA::TphoA fusion junctions (Fig. 1) revealed that all formed in-frame protein fusions between AP and the predicted PhoD, PhoE, and PhoT proteins. No TphoA insertions which expressed AP activity were located in the phoC gene. This is not surprising in view of the similarity between PhoC and the hydrophilic ATP-binding component of ABC transport systems; these proteins appear to be located in the cytoplasm, where they associate with the cytoplasmic membrane (22; see also reference 3). The three ndfA::TphoA insertions (2A, 10C and 1A, Fig. 1) which showed the highest level of AP activity were located in the phoD gene. Together with the predicted N-terminal secretory leader peptide of PhoD, these data are consistent with the proposed role of PhoD as a periplasmic binding protein.

Of the remaining five TphoA insertions, 5A and 8D fused AP to PhoE at amino acid residues 51 and 188, respectively, while 10B, 6G, and 9A fused AP to PhoT at amino acid residues 62, 239, and 397, respectively. Hydrophobicity plots of PhoE and PhoT proteins revealed that each of these proteins contains four “certain” and two “putative” transmembrane domains (Fig. 2) (10). Consistent with the positive-inside rule (53), if we assume that there are six transmembrane domains and that the N termini are in the cytoplasm, we calculate a lysine-plus-arginine-cytoplasmic-domain bias of 10 for PhoE and 8 for PhoT. AP fusion 5A in PhoE and fusions 10B and 6G in PhoT are located between the first and second predicted transmembrane domains (boxes 1 and 2 in Fig. 2). Therefore, these fusions to be external, the N termini of the PhoE and PhoT proteins must be located in the cytoplasm. When this topology is extended across the protein, we note that insertion 9A in PhoT is located in the second predicted periplasmic domain (between boxes 3 and 4 in Fig. 2). Insertion 8D fuses AP to PhoE. 4 amino acids from the C-terminal end of the predicted third transmembrane domain: we assume that this fusion extends into the periplasm. In comparing the derived topologies of PhoE and PhoT, it is evident that much of the difference between these two proteins resides in the 258-amino-acid periplasmic loop of PhoT, which is very large in comparison with the equivalent 76-amino-acid loop of PhoE (see regions between boxes 1 and 2 in Fig. 2).

**Phosphate and phosphonate phenotype of ndfF mutants.** The above results prompted us to examine the ability of wild-type *R. meliloti* and three ndfF mutants to utilize various sources of phosphorus for growth in MOPS-buffered minimal medium. We compared the parental strain 1021 with the insertion mutants Rm G490 (phoC490) and Rm G491 (phoT491) and the deletion strain Rm G439, in which a 12-kb region including the *phoCDET* genes has been removed. As expected, the wild type and mutants grew well in media containing 2 mM glycerol-3-phosphate or aminoethylphosphonate as P sources, all three mutants grew very poorly in media containing 2 mM P, as the P source (Fig. 3; data for *R. meliloti* Rm 490 and Rm 491 were similar). These results suggested that the mutant strains were defective in P, assimilation.

The deletion strain, Rm G439, grew poorly with aminomethyl- or methylphosphonate as the P source, while growth of the *phoC* and *phoT* insertion mutants, Rm G490 and Rm G491, on these phosphonates was similar to that of the wild type (Fig. 3 and data not shown). Limited DNA sequencing of regions outside the *phoCDET* genes, but within the region deleted in strain Rm G439, has revealed a gene homologous to the *phnM* gene of *E. coli* (9); the *phnM* gene product is believed to be part of an enzyme complex (C-P lase) which cleaves the C-P bond upon entry of the phosphonate into the cell. We assume that deletion of *phnM* (and perhaps other *phn* genes) in strain Rm G439 inactivates the C-P lase with the result that Rm G439 cannot degrade or grow in media containing aminomethyl- and methylphosphonate as P sources. The growth of the *phoC* and *phoT* mutants with aminomethyl- and methylphosphonate suggests that these compounds can be transported by an alternate system in *R. meliloti*. It is not yet clear whether the PhoCDET proteins are involved in phosphate uptake. In this respect, we note that *Enterobacter aerogenes* has two phosphate degradative pathways (27).

To determine the nature of the phosphate utilization defect in the ndfF mutants, we examined phosphate-starved cells of the wild type and the ndfF mutant Rm G439 for their ability to take up $^{33}P$. As a positive control, we examined the ability of the same cells to transport [14C]sucinate (Fig. 4). Unlike the parent strain Rm 1021, the ndfF mutant failed to transport phosphate, whereas both strains transported sucinate at similar rates. Together, the uptake, growth, and sequence homology data show that the *ndfF* locus encodes an uptake system which transports P, and possibly phosphonates, across the cytoplasmic membrane.

**Phosphate control of *ndfF* expression.** To determine whether expression of the *ndfF* locus responds to available phosphate, we employed Tn5-B20 (45) to generate transpositional gene fusions between lacZ and *ndfF* on the cosmids pTH21. The DNA sequences of the fusion junctions of four Tn5-B20 insertions which lay within the 7.3-kb BamHI restriction fragment were determined (Fig. 1). Insertions 7A and 19 were located in, and transcribed in the same direction as, the *phoD* and *phoE* genes, respectively. Insertion 4B was in the *phoT* gene, and the direction of transcription of *lacZ* was opposite to that of *phoT*. Insert 17 lay downstream of the *phoCDET* genes. Employing these plasmid-borne gene fusions in an *R. meliloti* Lac– background, we assayed for β-galactosidase activities in cells cultured in a MOPS-buffered minimal medium with added phosphate (2 mM) and without added phosphate (Fig. 5). We also measured AP activity, as this activity is known to be derepressed in P-limited cultures (46, 54). As expected, we observed high-level AP activities in all cells cultured in the absence of added P and low-level activity in cells grown in the media containing P (Fig. 5, open bars). The β-galactosidase activities of strains carrying insertion 4B
FIG. 2. Alignment of deduced amino acid sequences for R. meliloti (Rm) phoC, phoD, and phoET with the PnhC, PnhD, and PnhE proteins of E. coli (Ec) respectively. The * and - symbols below the sequences indicate residues which are identical and conserved, respectively, within the proteins. Boxes A and B in the R. meliloti PhoD-E. coli PnhD alignment indicate the conserved Walker motifs characteristic of many nucleotide-binding proteins. The N-terminal boxed region of R. meliloti PhoD indicates a potential secretory signal sequence. Boxed regions in the alignment between R. meliloti PhoE, R. meliloti PhoT, and E. coli PnhE indicate certain (boxes 1, 2, 3, and 6) and putative (boxes 4 and 5) membrane-spanning segments as predicted by Top Pre II (10).
Phosphorus Sources

FIG. 3. Histogram representing the growth of wild-type R. meliloth Rm 1021 (black bars), the phoCDT deletion mutant, Rm G45N (cross-hatched bars), and the phoCDT mutation mutant, Rm G48P (open bars), when supplied with various sources of P. MOPS-minimal medium contained no phosphate (PN), 2 mM P, (P2), 2 mM ammoniumphosphate (AMPN), 2 mM ammoniumphosphate (AMPN), 2 mM methylphosphonate (MPN), 2 mM ethylphosphonate (EPN), or 2 mM glycerol-3-phosphate (G3P). The optical density at 600 nm (OD600) measured after 60 h is reported as a percentage of the growth of strain 1021 in 2 mM P (100%). Values are the means of triplicate determinations.

or 17 were similar under the two growth regimes; however, the phoB and phoE gene fusions 19 and 7A were induced 10- to 25-fold in response to phosphate starvation (Fig. 5, black bars). Thus, expression of phoD and phoE is derepressed in response to limiting phosphate. The substantially lower level of β-galactosidase activity detected from the strain with the phoE gene fusion (fusion 19) in comparison with that for the phoD fusion (fusion 7A) suggests that these genes may be differentially expressed. In agreement with this suggestion, we note that an examination of the previously reported AP activities derived from strains carrying the pho::TaphoA insertions (Fig. 1) reveals that the levels of activity from strains with the phoD insertions (2A, 10C, and 1A) were at least three times higher than the activity from phoE or phoT insertion strains (3A, 8D, 10B, 6G, and 9A) (see Fig. 2 in reference 8). It is possible that the 172-bp phoD-phoE intergenic region has a role in regulating phoE and phoT expression.

To map the transcription start site of phoC, we extracted mRNA from cells grown in LBmc, as we have observed that the expression level of the phoD and phoE lacZ fusions (fusions 7A and 19) was high in LBmc-grown cells. In two separate experiments, we extended a primer from the 5' end of phoC (see Materials and Methods) and observed one major transcript which started 40 bp upstream of the translational start codon (Fig. 6). Analysis of this region revealed a possible -10 region preceded by two tandem 18-bp sequences, located at bp -23 to -40 and -45 to -62 relative to the transcriptional start site, which share 12 and 11 identical nucleotides, respectively, with the PHO box consensus CTGTCA(T/A) A(A,T)CTGTCA(C/T) of E. coli (33, 54).

DISCUSSION

The data in this paper demonstrate that the ndvF locus of R. meliloth consists of four genes, phoCDT, which together code for an ABC-type solute uptake system that transports phosphate and possibly phosphonates, across the cytoplasmic membrane. R. meliloth ndvF mutants form root nodules which fail to fix N₂ (Fix²⁻). In many of these mutant nodules, the infection process is blocked early, before release of the bacteria from the infection threads (8). Given that we now know that the ndvF locus encodes a phosphate transport system, and that ndvF mutants fail to grow in defined medium containing 2 mM H₂PO₄⁻ as the sole P source, it seems likely that the Fix²⁻ symbiotic phenotype results from an inability of the mutants to
FIG. 6. Promoter analysis of R. meliloti (Rm) phoC gene. The autoradiograph contact print shows the primer extension product (lane +1) 5 μg of RNA from LBme-grown strain Rm (1021) and the products of sequencing reactions with the same primer. The relevant sequence is shown on the left of the gel and the position of the extension product is indicated by an arrowhead. On the right is shown the promoter region of R. meliloti phoC. The transcription start site is indicated by +1, the −10 region is underlined, and the boxed sequences indicate the positions of the two putative PHO boxes.

grow during the infection process within the nodule. The ndvF mutants exhibit a delay in inducing root nodules in comparison with nodule development with the wild-type strain (8). This delay may also result from reduced bacterial growth, and thus require for a longer time period to reach a cell density such that sufficient nod factor signal is synthesized to trigger root nodule formation (30). There are other possible explanations for the symbiotic phenotype of the ndvF mutants: for example, the P status of the bacteria may be involved in the regulation of other cellular processes involved in the symbiosis. In this respect, it is interesting that in the plant pathogen Agrobacterium tumefaciens phosphatase starvation increases the expression of the central virulence regulatory gene, virG (55).

We are currently investigating the nature of the symbiotic defect by characterizing strains carrying second-site mutations which suppress the Fix phenotype of the ndvF mutants (38). The elucidation of the biochemical mechanism of suppression will clarify the role of the ndvF locus in nodule development.

The conclusion that the phoCDET genes encode a periplasmic binding protein-dependent system for the transport of phosphate and likely phosphates is based on (i) the high degree of homology of PhoC to the ATP-binding proteins of the ABC-type transport systems and the deduced topology of the PhoD, PhoE, and PhoF proteins; (ii) the fact that phoCDET mutants fail to grow in defined media containing 2 mM P; and (iii) the failure of the phoCDET deletion mutant, RmG439, to transport γ32P-labeled phosphate despite its ability to transport succinate at wild-type rates.

The inability of the R. meliloti phoCDET mutants to grow when P is supplied as 2 mM P suggests that the phoCDET genes encode the sole phosphate transport system in R. meliloti. Conversely, the growth of the phoCDET insertion and deletion mutants in media containing 2 mM glycerol-3-phosphate or amionophosphophote indicates that there are separate uptake systems for these compounds in R. meliloti. The existence of distinct phosphate and glycerol-3-phosphate uptake systems in many bacterial species is well-known (e.g., see reference 26), and E. aerogenes probably has two separate phosphatotransporters capable of aminationphosphophate transport (27). Data from uptake experiments employing cells of various Rhizobium species grown under P-limiting and P-excess conditions also led other workers to conclude that rhizobia contain a single, repressible, energy-dependent phosphate transport system (47). However, bacterial phosphate transport is best characterized in E. coli and Acinetobacter johnsonii, and both of these organisms contain two major p transport systems: one is a low-affinity system (pit) which is believed to be constitutively expressed, and the other is a high-affinity, binding protein-dependent system (phoSCAB) which is expressed under phosphate-limiting conditions (43, 50, 51). We believe that it is premature to conclude that R. meliloti contains a single phosphate transport system, as we recently identified a pit-like gene, and the expression and regulation of this gene are currently under investigation (4). The PhoCDET proteins of R. meliloti are clearly similar to the phosphate uptake proteins PhtDCE of E. coli (Fig. 2) rather than to the proteins of the phoSCAB-encoded high-affinity phosphate transport system of E. coli. The phosphate transport genes are cryptic in E. coli K-12-derived strains (34); however, when this system is active, there is good evidence that it can transport P, in addition to phosphates (36). Further experiments are required to definitively establish that the R. meliloti PhoCDET system also transports phosphates. In this respect, we note that in our study, all of the members of the family Rhizobiaceae examined were able to utilize methyl-ethyl-, aminomethyl-, and aminoethylphosphate as sole sources of P (32).

In view of the very low concentrations of soluble P, in most soils (0.1 to 10 μM) (6), it is possible that the acquisition of phosphate plays an important role in the growth and survival of soil microorganisms. In this respect, it is interesting that Beck and Munns (5) found a large variation in the ability of strains from various Rhizobium species to grow and survive at very low phosphate concentrations. Moreover, Almendras and Bottomley (1) and Leung and Bottomley (31) have presented strong evidence to establish a link between the phosphate-sequencing abilities of Rhizobium in foli strains and nodulation competition, as influenced by the addition of phosphate or lime to soil.

In E. coli, the PHO regulon consists of some 30 genes in
eight operons whose expression is derepressed under phospho-
limited conditions (43, 54). Transcription of these operons is regulated by the PhoB protein, which binds to similar 18-bp sequences (PHO boxes) in the regions of their promoters from positions -22 to -42. We have identified two tandem PHO-box-like sequences, separated at bp -23 to -40 and -45 to -52 relative to the transcriptional start site of the R. meliloti phoC gene (Fig. 6). The phoS and  ungB promoters in E. coli have similarly arranged PHO boxes also, with a 4-nucleotide gap between boxes. As expression of the phoD and phoE genes is induced over 10-fold in response to phosphate starvation (Fig. 5), it is likely that the two putative PHO boxes in the phoC promoter are functional. It is also likely that phoCD gene and the other genes which constitute the PHO regulon in R. meliloti, will be derepressed under the low-phosphate conditions found in soil.

The recognition that the phoCD gene encodes a phosphate transport system is also of interest, as this locus is focused on the 1,700-kb PEXO megaplasmid of R. meliloti. As in the case of genes involved in thiamine biosynthesis and carboxylase utilization, and the other genes located on this plasmid (7, 16), the phoCD gene is likely to be important in the life of the bacteria in the soil environment; however, they are clearly not essential for growth of the bacteria under all culture conditions.

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REFERENCES


2- **Specificity of the phosphate transport system encoded by phoCDET.**

To determine the affinity and the specificity of the PhoCDET phosphate transport system, kinetic constants were determined and competition studies were performed. Phosphate uptake over a 5 minute period showed that the phoCDET deletion mutant RmG439, starved for phosphate, was unable to transport phosphate (Pi) when 10μM $^{33}$P-orthophosphate was provided in the medium (see Fig. 4; Bardin et al., 1996). The kinetic constants of the wild type strain Rm1021, grown under phosphate limitation, were then determined using concentrations of $^{33}$P-labeled orthophosphate from 0.05 to 3μM. When the data was plotted using [S] versus v axes, a typical Michaelis-Menten curve was obtained (Fig. 3-1). A Km value of 0.21μM and a Vmax value of 6.7 nmol Pi/min/mg protein was determined using a Hanes-Woolf plot (Fig. 3-2). The very low Km value obtained for Pi uptake indicated that phoCDET encodes a high affinity phosphate transport system, similar to pst, the high affinity phosphate transport system of *E. coli* (Km: 0.4μM; Willsky and Malamy, 1980a).

The sequence homology between the phosphonate transport system of *E. coli* (phnCDE) and phoCDET indicates that ndvF may also be involved in phosphonate uptake (Bardin et al., 1996). The specificity of PhoCDET for phosphate was addressed by performing experiments investigating the inhibition of phosphate uptake by various phosphonate compounds. Inhibition experiments
Fig. 3-1: Michaelis-Menten curve showing phosphate uptake of phosphate-starved wild type strain when concentrations between 0 and 3μM of $^{33}$P-labeled phosphate were provided to the reaction mixture. Each point represents the average of triplicate value ± standard error (error bars).
**Fig. 3-2:** Hanes-Woolf plot used to determine the kinetic values of phosphate uptake by the PhoCDET system. Each point represents the average of triplicate values. The linear regression was calculated to:

\[ Y = 0.148348 \times (X) + 0.031835 \] with correlation coefficient of \( r^2 = 0.996114 \).
by arsenate were also performed as this phosphate analogue was shown to compete with phosphate for transport in many organisms such as *E. coli* (Bennett and Malamy, 1970), *Bacillus cereus* (Rosenberg et al., 1969), *Micrococcus lyzodeikticus* (Friedberg, 1977) and *Streptococcus faecalis* (Harold and Spitz, 1975). Inhibitors were provided at 5 (5\(\mu\)M) and 50 times (50\(\mu\)M) the concentration of labeled phosphate (1\(\mu\)M).

Cold phosphate at 5 times the labeled-phosphate concentration showed 82% uptake inhibition as expected (Table 3-1). Succinate was used as a negative control and showed no phosphate uptake inhibition. Ethylphosphonate (EP), aminoethylphosphonate (AEP) and methylphosphonate (MP) inhibited phosphate uptake to a greater extent than phosphate itself (88 to 96% phosphate uptake inhibition at 5 times the \(^{33}\)Pi concentration). If this is due to competitive inhibitions, our results suggest that EP, AEP and MP have a higher affinity for PhoCDET than phosphate and may be transported into the cells by this transport system. Aminomethylphosphonate (AMP) showed less inhibition activity than the other phosphonates with 60% uptake inhibition when provided at 5 times the concentration of labeled-phosphate. This may be attributed to structural constraints of this compound on the binding site of the transporter. It was interesting that the phosphonate, glyphosate [\(N\)-(phosphonomethyl)glycine], the active ingredient in the herbicide Roundup, showed very little phosphate uptake inhibition even when provided at 50 times the concentration of labeled
% inhibition of Pi transport via PhoCDET

<table>
<thead>
<tr>
<th>Competitors</th>
<th>5μM</th>
<th>50μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Pi</td>
<td>82.4 ± 1.6</td>
<td>97.9 ± 0.5</td>
</tr>
<tr>
<td>AEP</td>
<td>96.1 ± 1.3</td>
<td>98.3 ± 1.7</td>
</tr>
<tr>
<td>EP</td>
<td>94.5 ± 1.1</td>
<td>99.7 ± 0.3</td>
</tr>
<tr>
<td>AMP</td>
<td>57.8 ± 4.8</td>
<td>92.4 ± 0.9</td>
</tr>
<tr>
<td>MP</td>
<td>88.0 ± 2.7</td>
<td>99.7 ± 0.3</td>
</tr>
<tr>
<td>glyphosate</td>
<td>7.8 ± 3.0</td>
<td>17.1 ± 0.6</td>
</tr>
<tr>
<td>arsenate</td>
<td>77.5 ± 1.9</td>
<td>98.4 ± 0.2</td>
</tr>
<tr>
<td>Succ</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Phosphate uptake was performed at $^{33}$Pi concentration of 1μM. Rate of uptake with no inhibitor present was 5.5 ± 0.2 nmol/min/mg protein.

Table 3-1: Inhibition experiments investigating the specificity of PhoCDET for phosphate. The experiments were performed on phosphate starved cells and the numbers reported in the table express the percent inhibition when 1μM $^{33}$P-labeled phosphate was provided to the medium. The inhibitors (at 5 or 50 times the concentration of $^{33}$P-labeled phosphate) were added to the cells 15 sec prior to the addition of the radio-labeled compound. The inhibitors used were cold orthophosphate (Pi), ethylphosphonate (EP), aminoethylphosphonate (AEP), methylphosphonate (MP), aminomethylphosphonate (AMP), glyphosate, arsenate and succinate (Succ). The data were from a 1 min uptake experiment and the % inhibition, representing the average of three independent assays, was calculated from a Pi uptake rate of 5.5 nmol/min/mg protein obtained in the absence of inhibitors.
phosphate (17% inhibition). The ability to degrade glyphosate and the use of this compound as a phosphorus source was shown to be widespread in the Rhizobiaceae family (Liu et al., 1991). The inhibition experiments presented here as well as the ability of R. meliloti to grow in MOPS-buffered minimal media containing glyphosate as the sole phosphorus source (Liu et al., 1991) suggest that glyphosate uptake does not involve PhoCDET. Arsenate inhibited phosphate uptake similarly to cold phosphate (77% inhibition was observed when arsenate was provided at 5 times the labeled phosphate concentration) suggesting that this phosphorus analogue may be transported into the cell by the PhoCDET system with an affinity similar to that for phosphate.
C. Discussion

The ndvF locus is made up of four genes, phoCDEF that together encode an ATP-binding-cassette dependent transport system responsible for the uptake of phosphate and possibly phosphonates. The presence of two Pho Boxes upstream of phoC and the 10- to 20-fold induction in level of expression of the system under phosphate deprivation suggest that this locus is part of a Pho regulon in R. meliloti similar to the Pho regulon described in E. coli (Wanner, 1993).

The kinetics of phosphate uptake into cells grown under phosphate limiting conditions indicate the presence of a single high affinity (Km: 0.2μM), low velocity (Vmax: 6.7 nmol Pi/min/mg protein) phosphate transport system. The Km value measured was significantly lower (8- to 30-fold) than the Km values for phosphate uptake reported for seven rhizobia/bradyrhizobia strains (Smart et al., 1984a) but was similar to the value of the high-affinity phosphate transport system (pst) of E. coli (Km: 0.4μM; Willsky and Malamy, 1980a), Pseudomonas aeruginosa (Km: 0.46μM; Poole and Hancock, 1984), and Acinetobacter johnsonii (Km: 0.7μM; Van Veen et al., 1993a). In the study of Smart et al. (1984a), the single strain of R. meliloti examined (RmWU3) had a Km of 6μM. This value is very similar to the Km value of the pit-like alternative phosphate transport system in R. meliloti 1021 (see Chapter IV; Dr. Voegele,
personal communication) and to the low-affinity Pit phosphate transport system of *E. coli*. In addition, although Pi uptake by RmWU3 was unidirectional, the system was not sensitive to osmotic shock suggesting that the phosphate transport system studied in RmWU3 was not a periplasmic binding protein-dependent transport system like the ABC-type transporter. These observations indicates that (a) *R. meliloti* WU3 strain only carried the low affinity-type phosphate transporter or (b) under the growth and transport assay conditions used by these authors, only the low-affinity phosphate transport system was expressed. The second proposition is favored as a fragment homologous to the 7.3kb *BamHI* fragment (containing the *phoCDET* locus) of *R. meliloti* 1021 was found in at least three other *R. meliloti* strains (Charles et al., 1991), suggesting that *phoCDET* is conserved among *R. meliloti* strains.

The Vmax value of the PhoCDET system was low, even when compared to the low velocity of the *pst* system of *E. coli* (Vmax: 15.9 nmol Pi/min/mg protein; Willsky and Malamy, 1980a). This may be due to the slower growth rate of *R. meliloti* compared to *E. coli*. It is important to mention that slight variations in growth conditions lead to variations in the velocity of phosphate uptake. We observed variations from 3 to 6.7 nmol Pi/min/mg protein in the Vmax's determined from different experiments in which P starved cells were examined.

Phosphate uptake via the PhoCDET transport system was inhibited by the presence of arsenate and phosphonate compounds. These compounds may act
as competitors for phosphate uptake, either by binding to the periplasmic Pi-binding protein thus making it unavailable for phosphate uptake or by being transported into the cell by the PhoCDET transport system. The periplasmic Pi-binding protein of the PhoCDET system, encoded by *phoD*, may then have a broad specificity for various phosphorus compounds. This would then be in contrast with the Pst system of *E. coli* in which the periplasmic Pi-binding protein, encoded by *pstS*, is highly specific for phosphate and does not even recognize the phosphate analogue arsenate as a substrate (Bennett and Malamy, 1970; Medveczky and Rosenberg, 1971).
Appendix A

Further characterization of the growth of *phoCDET* mutants on phosphonate and phosphorus compounds.

Growth curves of the wild type strain (Rm1021, ■) and the three *phoCDET* mutants; RmG439 (*ndvFΔ439*, ▲), RmG490 (*phoCΩ490*, ●) and RmG491 (*phoTΩ491*, ◆) in phosphate-free MOPS-buffered minimal media (P0), and containing 2mM of, orthophosphate (Pi), aminoethylphosphonate (AEP), ethylphosphonate (EP), aminomethylphosphonate (AMP), methylphosphonate (MP), glycerol-3-phosphate (G3P), glucose-6-phosphate (Glc6P) or phosphoserine (PSer). The cells were inoculated in the various media after a 24 hour period of growth in MOPS P0.

Analysis of the results

- The four strains grew poorly in MOPS P0.
- In MOPS Pi, the wild type reached saturation after 65 hours growth (doubling time: 5 hours); The three *phoCDET* mutants behaved similarly with a slow but
constant growth (doubling time: 30 hours), reaching the wild type growth saturation level after 160 hours.

- In MOPS AEP, the four strains grew at similar rate.

- In MOPS EP, the wild type growth was delayed by 40 hours compared to its growth in MOPS Pi. The three mutants behaved similarly with a slow and constant growth similar to the one observed in MOPS Pi.

- In MOPS AMP and MP, RmG490 and RmG491 behaved similarly but were different from RmG439: RmG439 grew poorly in both media while both RmG490 and RmG491 grew as well as the wild type strain in MOPS AMP and reached the wild type level of growth after a 20 hours delay in MOPS MP.

- In MOPS G3P, Glc6P and PSer, the three phoCDET mutants grew at similar rate to that of the wild type. Growth of the wild type strain was delayed by 40 hours in MOPS Glc6P compared to its growth in MOPS Pi.
CHAPTER IV

Genetic characterization of the sfx1 locus

A- Introduction

The ndvF locus of R. meliloti contains four genes designated phoCDET. These genes were found to encode a phosphate transport system and the Fix\(^-\) phenotype of ndvF mutants when inoculated on alfalfa plants was attributed to their inability to assimilate enough phosphate for growth during nodule formation process (Chapter III; Bardin et al., 1996). It was also observed that occasionally, plants inoculated with the Fix\(^-\) phoCDET strains formed some pink Fix\(^+\) nodules (Charles et al., 1991). Bacteria isolated from these nodules carried the original phoCDET mutations and genetic analysis revealed that these strains also carried second-site mutations (designated sfx) which suppressed the Fix\(^-\) phenotype. The sfx mutations in five independent pseudorevertant strains were shown to map to two distinct loci (Oresnik et al., 1994). Class I suppressor mutations (sfx1, sfx4 and sfx5) were tightly linked to the insertions \(\Omega5117::\text{Tn5}\) and \(\Omega5122::\text{Tn5-132}\) while class II suppressor alleles sfx2 and sfx3 were not. The class II suppressor strains, but not class I, were sensitive to deoxycholate, sodium
dodecyl sulfate and sarkosyl as well as to the antibiotic bacitracin (Oresnik et al., 1994).

The sfx1 locus was cloned from a pRK7813 cosmid library containing partial BamHI DNA fragments from the class I suppressor strain RmF263 (ndvF, sfx1) (Oresnik et al., 1994). Cosmids pTH56 and pTH57 which suppressed the Fix⁻ phenotype of phoCDET deletion mutants were isolated. Both cosmids shared an identical 18kb BamHI fragment, and direct evidence that the insert DNA in pTH56 was collinear with the sfx1 region was obtained by Southern blot analysis revealing that some transposon insertions linked in transduction to the sfx1 region lay within the pTH56 insert DNA.

This chapter presents a molecular genetic characterization of the sfx1 locus and presents data addressing the physiological mechanism through which sfx1 suppresses the Fix⁻ phenotype of ndvF mutants.
B- Material and Methods

1- Construction of transcriptional lacZ fusions to pit and orfA

Plasmid-borne lacZ fusions to the pit open reading frame were made by subcloning the 2.1kb EcoRI fragments from pTH354 (wild type) and pTH380 (sfx1) into the EcoRI restriction site of the pMP220 vector (Spaink et al., 1987) to create plasmids pTH376 and pTH365. Fusions of the "orfA" open reading frame to lacZ were made by deleting the DNA between the SphI site located in the orfA gene and the SphI site of the pMP220 polylinker of plasmids pTH376 and pTH365 to create plasmids pTH378 and pTH367, respectively. The plasmid fusions were then mated into wild type and phoCΩ490, Lac^- strains (RmG212 and RmH667, respectively).

Chromosomal lacZ gene fusions to pit in both wild type and sfx1 backgrounds were constructed as outlined in Fig 4-1a. The lacZ cassette was inserted, in both orientations, immediately downstream of the pit translational stop codon so that the pit product would be functional. The resulting pBR322 based plasmids, pTH351 and pTH352, were recombined by a single crossover into the genome of wild type or sfx1, Lac^- background strains. Spectinomycin resistant recombinants were checked for resistance to 2μg/ml Tc and by Southern blot using pTH276 as probe (Fig. 4-1b).
Fig. 4-1a: Construction of the pit chromosomal lacZ fusions was performed as follows:

a) The 0.5kb EcoRI fragment (R₁-R₂) of pTH90 (containing the 3'-end of sfx1-pit) was subcloned in pUC118 so that R₁ was on the polylinker side. The subclone was digested with SacII (located 11 nucleotides upstream from the pit translational stop codon) and PstI (located in the polylinker) and the fragment was replaced by a linker (with SacII/ PstI protruding ends) to create pTH343. The linker was constructed by hybridizing two complementary oligos. The two oligos (5'-GGACCTCGTCCCTGACGCGGCTGCA-3' and 5'-GCCCGGGTCAGGGCGACGAGGTCCGC-3') were synthesized so that the end of the pit gene was reconstituted (in order to obtain a functional gene). The translational stop codon was followed by a Smal site used to clone the lacZ cassette. pTH343 was then digested with PstII/EcoRI and the fragment was cloned in pBR322 digested with the same enzymes to create pTH344. The lacZ cassette of the pLMS clone (pUC18 with the lacZmobSp' cassette) was subcloned as a Smal fragment in pTH344 to create pTH351 and pTH352 with the lacZ gene in the same and opposite orientation as pit, respectively.

b) The plasmids were recombined into the chromosome of a wild type and a sfx1 strain by single cross-over using homologous recombination of the 0.3kb SacII/EcoRI(2) fragment.

Symbols used: poly: polylinker; H: HindIII; Pt: PstI; R: EcoRI; SclI: SacII; Sm: Smal.
Fig. 4-1b: Southern blot of Rif$^\text{r}$ Sp$^\text{r}$ recombinants obtained following transfer of pTH351 and pTH352 into the wild type strain Rm5000 and probed with the pTH276 (HindIII/Sacl, 4.8kb) plasmid. Line 1, 4 and 7: Rm5000-pTH351 recombinant; Line 2, 5 and 8: Rm1021; Line 3, 6 and 9: Rm5000-pTH352 recombinant. Digestion with EcoRI (R): Line 1, 2 and 3; Digestion with HindIII (H): Line 4, 5 and 6; Digestion with Sacl/HindIII (Sc/H): Line 7, 8 and 9. Line a: 1kb ladder. The figure on the following page shows the fragments expected for each recombinant type as well as for the wild type strain.
Rm5000 - pTH351 recombinants

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Wild type

---

Rm5000 - pTH352 recombinants
2- Construction of pTH396 and pTH397

These constructs were prepared to delineate the fragment in which the sfx1 mutation was located. As the mutation was suspected to be in the promoter region of orfA, the 920bp XhoI/EcoRI(3) and 540bp EcoRV/EcoRI(3) fragments (see Fig. 4-4a) of pTH191 were subcloned as XhoI/HindIII and EcoRV/HindIII fragments (using the HindIII site of the polylinker) into the SalI/HindIII and EcoRV/HindIII restriction sites of pBR322, respectively. The pBR322 vector was chosen because it is mobilized by pRK600 but cannot replicate in Rhizobium meliloti. To provide a selective marker to identify R. meliloti recombinants, the 3.4kb HindIII fragment of pGS220 containing the KmR gene of Tn5 (De Vos et al., 1986), was subcloned into the HindIII site of the two subclones.

3- DNA sequencing and sequence analysis

(see also material and methods)

DNA sequencing was performed on single-stranded DNA using the dideoxy chain termination method according to the protocol of United States Biochemicals for the Sequenase 2.0 enzyme and using [α-35S] ATP (NEN/DuPont). Single stranded DNA was obtained from host strain XL-1Blue (Stratagene) following infection with the helper phage M13K07 (Vieira, and Messing, 1987). Both strands of DNA were sequenced using the -20 (lacZ) primer (5’-GTAAACGACGGCCAGT-3’) and the IS50 primer (5’-TCACATGGAA
GTCAGATCCT-3'). DNA and deduced protein sequences were analyzed with PC/Gene (Intelligenetics), Blast (Gish and States, 1993), CLUSTAL W (Thompson et al., 1994) and Top Pred II (Claros and von Heijne, 1994) software.
C. Results

1. *sfx1* suppresses the growth phenotype of *phoCDET* mutants in 2mM Pi

To investigate whether *sfx1*-mediated suppression of the Fix phenotype of *phoCDET* mutants was related to phosphate assimilation, *sfx1* was tested for its ability to suppress the poor growth phenotype of *phoCDET* mutants in MOPS-buffered minimal media containing 2mM phosphate (MOPS P2). An *sfx1* *phoCΩ490* double mutant, strain RmG762, was constructed by transducing the ΩSp insertion from strain RmG490 (*phoCΩ490*) into strain RmG591 (*sfx1*). The growth of Rm1021 (wild type), RmG490 (*phoCΩ490*) and RmG762 (*phoCΩ490, sfx1*) in 2mM phosphate was monitored over a 160 hour time period and is presented in Fig. 4-2a. The wild type strain had a doubling time of 5.4 hours in the exponential phase of growth and reached the stationary phase 65 hours after inoculation. The *phoCΩ490* mutant showed a slow but constant growth rate (doubling of 34.8 hours between OD600 of 0.1 to 0.6) and reached growth saturation 160 hours after inoculation. An identical growth pattern was obtained for the *phoCDET* deletion strain (*ndVFΔG439*) and for *phoT* mutant (*phoTΩ491*) (see Appendix A). The presence of the *sfx1* mutation in the *phoCΩ490* strain restored its growth rate to near the wild type (doubling time 6.6 hours). *sfx1*
Fig. 4-2a: Growth of Rm1021 (wt, ■), RmG490 (phoCΩ490, □) and RmG762 (phoCΩ490, sfx1, ★) in MOPS-buffered minimal media supplemented with 2mM Pi. Each time point represents the average of triplicate values.
**Fig. 4-2b:** Growth of the wild type strain and the *phoC*Δ490 mutant when increasing phosphate concentrations were added to the media. The growth of Rm1021 was similar in every media (■). The growth of *phoC*Δ490 is reported as follows: 2mM (●), 10mM (Δ), 20mM (▼) and 60mM Pi (☆). Each time point represents the average of triplicate values.
was also able to suppress the growth phenotype of \textit{ndvFΔG439} and \textit{phoTΩ491} in a similar manner (data not shown).

The \textit{sfx1}-dependent suppression of the growth phenotype of \textit{phoCDET} mutants in media containing 2mM Pi provided further evidence that the Fix\textsuperscript{−} phenotype of these mutants was due to poor phosphate assimilation. Suppression by \textit{sfx1} seems to occur by providing the mutant cells with an alternative and efficient way to assimilate phosphate. The slow growth of \textit{phoCDET} mutants in MOPS media containing 2mM Pi suggests that an alternative transport system(s) with a low affinity for phosphate and independent from the \textit{phoCDET} system is present in the cell.

When growth of the \textit{phoCΩ490} mutant in media containing increasing concentrations of phosphate was examined, the growth rate increased with increasing Pi; in 60mM Pi the mutant strain reached a doubling time in the exponential phase that was 80\% of that of the wild type strain (Fig. 4-2b). Thus other transport systems able to transport phosphate appear to be present in \textit{Rhizobium meliloti}. However the low affinity of such system(s) for phosphate suggests that they probably don't play an important role as phosphate transporter in a wild type cell.
2- Localization of sfx1 on pTH90

The sfx1 locus was previously localized to an 18kb BamHI fragment of the pTH56 cosmid (Oresnik et al., 1994). A 12kb HindIII fragment, internal to the 18kb BamHI fragment of pTH56, was subcloned into pRK7813 to give pTH90. Transconjugants obtained following transfer of pTH90 into RmG490 (phoCΩ490) formed Fix⁺ nodules and grew like the wild type strain in media containing 2mM phosphate (Fig. 4-3b, 4-3c). These data confirmed that pTH90 possessed the entire sfx1 locus. A restriction map of pTH90 was constructed for the enzymes EcoRI, BglII and ScaI. To localize the sfx1 region 19 Tn5 insertions which mapped within the 12kb insert of pTH90 were isolated (Fig. 4-3a). Using seven of these Tn5 insertions, we created defined deletions of pTH90 by removing DNA from the BamHI restriction site within the polylinker to the BamHI site within the Tn5 insertions. The resulting plasmids were mated into RmG490 (phoCΩ490) and the Tc⁺ transconjugants were tested for suppression of the mucoid phenotype on low osmolarity media (GYM). Deletions removing any of the 7kb region up to insertion ωE suppressed the mucoid phenotype of RmG490 while further deletions prevented suppression (Fig. 4-3b). In addition, while the Fix⁻ phenotype of the phoCΩ490 strain was suppressed by either pTH90 or pTH90ΔωE, phoCΩ490 strains containing the pTH90 plasmids deleted up to insertion ω2.3, ω3.10 and ω2.2 remained Fix⁻ (Fig. 4-3b). As these data suggested that the sfx1 suppressor locus lay within the 4.8kb HindIII-ScaI
**Fig. 4-3a:** Restriction map of pTH90 showing the location of the restriction sites for Bg: BglII; H: HindIII; R: EcoRI and Sc: ScaI. Also shown are the locations of 19 Tn5 insertions (●).

**Fig. 4-3b:** (next page) BamHI (B) deletions of seven pTH90::Tn5 insertions (Ω2.15; 1.4; 1.5; E; 2.3; 3.10 and 2.2). The table on the right side of the figure indicates the osmolarity on glutamate/yeast/mannitol (GYM) media and the Fix phenotypes of RmG490 (phoCΩ490) into which the truncated plasmids were mated. In GYM/NaCl, NaCl was supplemented at a concentration of 100mM. D and M indicate a dry and mucoid colony morphology types, respectively. The Fix phenotype was determined by the shoot dry weight in mg (average ± S.E. of 30 plants from 3 pots). The percent % dry weight was calculated in relation to that of Rm1021 (100%). + and - indicates effective and ineffective nitrogen fixation, respectively. UI: uninoculated, ND: not determined.
Fig. 4-3c: Restriction map and subclones of pTH276. The restriction enzyme shown are Bg: BglII; C: Clal; H: HindIII; R: EcoRI; Sc: SacI; Sm: SmaI; Sp: SphI; V: EcoRV and X: XhoI. Also indicated is the position of the pLac promoter of the vector relative to the orientation of the clones. The table on the right side of the figure indicates the growth (absorbance at OD600 after 60 hours growth) of the phoCΩ490 strain containing the different plasmids in MOPS-buffered minimal media supplemented with 2mM Pi. The values represent an average of triplicate data ± S.E.
fragment, this fragment was subcloned into pRK7813 and a restriction map for the enzymes EcoRV and SmaI was determined (Fig. 4-3c). In addition, the 2.6kb HindIII-EcoRV(1), the 2.5kb HindIII-SmaI(1) and the partial 2.6kb EcoRI fragments were subcloned in pRK7813 to give the plasmids pTH305, pTH304, pTH347 and pTH348 with pTH347 and pTH348 representing the two orientations of the 2.6kb EcoRI fragment (Fig. 4-3c). Plasmids pTH276, pTH305, pTH347 and pTH348 but not pTH304 allowed phoCΩ490 to grow on media containing 2mM phosphate suggesting that the sfx1 locus spanned the SmaI restriction site. From these data we deduced that the entire sfx1 locus was located between the EcoRI(1) and EcoRV(1) sites.

Seven Tn5 insertions and one TnphoA insertion located in this area were recombined onto RmG762 (phoCΩ490, sfx1) (Fig. 4-4a). The recombinants, checked by Southern blot using pTH276 as probe, were assayed for growth in MOPS media containing 2mM Pi. Recombinants for insertions Ω2.2 and Ω12 grew in this media as well as the wild type and thus retained the suppressor phenotype (Fig. 4-4b). Ω3.16, 3.3, J, 2.5, 3.10, 2.3 or 10A recombinants failed to grow in the 2mM phosphate media and thus disrupted the suppressor locus. As expected, recombinants Ω3.10 and Ω2.3 were also unable to restore the symbiotic phenotype of phoCΩ490 when tested on alfalfa plants (data not shown). Together, these results indicates that sfx1 is located between the EcoRI and the EcoRV(1) restriction sites.
Fig. 4-4a: Map showing the position and size of the two complete and two partial open reading frames deduced from the sequence of the fragment between EcoRI(3) and insertion Ω2.2 of pTH90. Homology of orfB for pit and orf1 for recF are indicated. Also indicated is the position of seven Tn5 insertions that disrupted the sfx1 locus (▼) as well as one Tn5 (●) and one Tn phoA (■) insertion located outside the suppressor locus. The restriction sites indicated are Bg: BglII; C: ClaI; H: HindIII; R: EcoRI; Sm: SmaI; Sp: SphI; V: EcoRV and X: Xhol.
Fig. 4-4b: Growth of Rm1021 (wt, □), RmG490 (phoCΩ490, ●), RmG762 (phoCΩ490, sfx1, ★), RmG821 (phoCΩ490, sfx1Ω2.2::Tn5, △), RmG822 (phoCΩ490, sfx1Ω2.3::Tn5, ◆), RmG830 (phoCΩ490, sfx1Ω3.10::Tn5, ▽) and RmH842 (phoCΩ490, sfx1Ω12::TnphoA, ▼) in MOPS-buffered minimal media supplemented with 2mM Pi. Each time point represents the average of triplicate values.

Note: the growth phenotypes of phoCΩ490, sfx1::Tn5Ω 3.16; 3.3; J; 2.5, and 10A were similar to RmG822 and RmG830. These results are not included in this figure.
Disruption of the sfx1 locus not only failed to suppress the growth phenotype of the phoCΩ490 mutant, it reduced its growth rate to 75.4 hours between OD_{600} of 0.1 to 0.6 (Fig. 4-4b). This locus in RmG490 appeared then to encode one of the alternative low affinity phosphate transport systems that allowed slow growth of the phoCΩ490 mutant in MOPS P2. Because some growth was still observed when both the sfx1 locus and PhoCDET systems were not functional, other alternative phosphate transporter(s) are probably present in the cell.

3- Nucleotide sequence of sfx1

The 2828bp nucleotide sequence from the EcoRI(3) restriction site to the Tn5 insertion Ω2-2 was determined (Fig. 4-4a). Both strands were sequenced by the dideoxy-chain termination method using overlapping fragments (see Appendix B-2 and 3 for strategy of sequencing and sequence). Sequence analysis revealed the presence of two complete (orfA and pit) and two partial (recF and orf2) open reading frames (ORF) (Fig. 4-4a). orfA and pit were transcribed in the same direction and encoded proteins of 214 (23.8 kD, pl: 4.57) and 334 (35.2 kD, pl: 8.89) amino acid, respectively. The adenosine nucleotide from the orfA stop codon (TGA) and the pit start codon (ATG) was common to the two genes suggesting that both genes were transcribed as a single transcript. In both cases, the ATG was preceded by a potential ribosome binding
site (GATGGA-N6-ATG for orfA and GAGA-N7-ATG for pit). A cruciform-type stem-loop structure (GC-rich) followed by seven AT-rich residues downstream of the pit stop codon (TGA) suggested the presence of a rho-independent terminator. Analysis of the region upstream from the orfA start codon revealed a potential Pribnow box, TACAAT (consensus sequence TATAA{T} (Pribnow, 1975; Schaller et al. 1975)) located 40 nucleotides away from the ATG site. The -35 region, whose consensus sequence reads tcTTGACat (Takanami et al., 1976; Seeburg et al., 1977), was not found 17 residues upstream from the TATA box. However we found a modified Pho Box-like sequence 35 nucleotides upstream from the putative TATA box (Fig. 4-5). This Pho Box-like sequence contained 7 mismatches compared to the E. coli consensus sequence CTGTCATAa(t)/At(l/a)CTGTCAC(t) (Wanner, 1993). Analysis of the promoter region of the E. coli pit gene, suggested to Sofia et al. (1994) that pit expression may be σ^{54} (ntrA)-dependent. To investigate whether expression of the orfA pit locus of R. meliloti was ntrA-dependent, pTH376 (pit::lacZ fusion) and pTH378 (orfA::lacZ fusion) were mated into RmH808 (ntrA, Lac^- strain) and assayed for β-galactosidase activity after 38 hours growth in MOPS P0 and MOPS P2. pit and orfA expression in this background showed no difference with their expression in a wild type background (data not shown). Thus, the orfA and pit genes do not require ntrA for expression.
**EcoRV**

GATATCGACGTTGCTGTCAGGTAGGATGAAAGCCCGGCA
-185

AGGCAAGGCGGAAAGGGGTGTTCTGTCCGCCTCTCCCCGC
-145

CTGCCGCAGTTCCGTATCCGGGCTGCGCATTTCGCCTCA
-105

? Pho Box?

ATCGCCTGTGGGAAAGCCGTTTTTCGGCCGCATCGGAAC
-65

CTGTCATAAAAACGTGCA

TTTCCCCGAGCGCGCAAGTACAATATGACAACTGCCTGAC
-25

AATCGACAGTTGGATGGATCGCTGCTCGCCCTGTTTC
+16

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**Fig. 4-5:** *sfx1-orfA* promoter region. Indicated are the orfA ATG codon (boxed, with A numbered as +1), the direction of translation (arrow), the ribosome binding site (dashed box), the potential TATA Box (bold italic) and the potential Pho box (dashed overline) beneath which the consensus Pho Box of an *E. coli* promoter is reported (bold). The *EcoRV* site is also indicated as well as the hexa-thymidine region of an *sfx1* sequence (double underline).

Note; in the wild type sequence, this region is made of **seven** thymidines.
Both orfA and pit had a high G + C content (61.8% for orfA and 63.9% for pit) with a strong bias for G and C at the third position (82.7% for orfA and 87.1% for pit) as expected for G+C rich organisms (Muto and Osawa, 1987). Comparison of the orfA and pit deduced proteins with the Rhizobium codon usage obtained from the analysis of 126 genes (Wada et al., 1992) revealed that few rare codons were present in these proteins, suggesting that these genes are probably expressed in R. meliloti.

recF was located 233 residues upstream from orfA and was transcribed in the opposite direction relative to orfA-pit. orf2 located 113 residues downstream from the pit stop codon was transcribed in the same direction as orfA-pit. A potential ribosome binding site was found upstream from both partial open reading frames; a TCGGA sequence was found 6 residues upstream from the ATG of recF and a GGAGGG sequence was located 5 nucleotides away from the ATG codon of orf2. Neither recF nor orf2 were part of the sfx1 locus because recF laid upstream from the EcoRV restriction site and was disrupted by the TnphoA(12) insertion and orf2 overspanned the EcoRI (2) restriction site and was disrupted by the Tn5 (2-2) insertion.
4- Analysis of the deduced proteins

The analyses of the 2828bp sequence encompassing the sfx1 locus, revealed the presence of two complete genes, orfA and pit, and parts of two genes designated recF and orf2.

a) The Pit protein

BlastX GenBank searches (Gish and States, 1993) revealed the Pit protein was similar to:

- the low-affinity Pit phosphate transport protein of E. coli (EcPitA) (Sofia et al., 1994). The Pit protein also showed similarity to numerous uncharacterized proteins homologous to EcPitA; these included EcPitB of E. coli (Bollinger et al., 1995) whose gene is located at 67 min on the chromosomal map and shares 81.0% amino acid identity with EcPitA, and the putative low-affinity inorganic phosphate transporter of Streptomyces halstedii (Blanco et al., 1993).
- the phosphate-repressible phosphate permease, of Neurospora crassa (NcPho-4+) (Mann et al., 1989) and homologous proteins such as several retrovirus receptors in human (vanZeijl et al., 1994), mice (Johann et al., 1992), hamster (Wilson et al., 1994) and rat (Miller et al., 1994).
- the glvr-1 deduced protein of Mycobacterium leprae (Smith, unpublished).
• the HI1604 deduced protein of *Haemophilus influenza* (a purple bacteria of the gamma subdivision; Fleischmann et al., 1995).

• a putative phosphate permease of *Arabidopsis thaliana* (Quigley et al., 1996).

A *pit*-like gene product appears to be widespread among both prokaryotic and eukaryotic organisms. Its function as a phosphate transporter has been characterized for EcPitA (Rosenberg et al., 1982), NcPho-4* (Mann et al., 1989), and some of the retrovirus receptors (see for review, Kavanaugh and Kabat, 1996).

Pairwise alignments revealed that EcPitA (499 aa), NcPho-4* (590 aa) and HI1604 (420 aa) shared 34%, 32% and 27% amino acid identity with the *sfx1*-Pit protein, respectively (Fig. 4-6a). Multiple alignment between these proteins (Fig. 4-6b) showed greatest similarity in the N and the C-terminal regions, whereas the central, mostly hydrophilic domain present in EcPitA and NcPho-4* and mostly hydrophobic domain of HI1604 was absent from RmPit. The strongest identity to Pit was obtained with the *givr-1* (*pit*) deduced protein of *Mycobacterium leprae* (414 aa) (39% identity, 61% conserved; Fig. 4-6a). Similarity was seen throughout these proteins except MIPit which carried an additional hydrophilic C-terminal extension 81 amino acids long. Alignment of these two genes revealed 63% identity at the nucleic acid level (Fig. 4-6c).

Hydropathy plot determined by the Tmpred and TopPred II programs and the positive-inside rule (von Heijne, 1986) confirmed the homology data in that
Fig. 4-6a: Pairwise alignments between the pit deduced protein of R. meliloti (RmPit) and, the pitA deduced protein of E. coli (EcPitA), the pho-4* deduced protein of N. crassa (NcPho4), the HI1604 deduced protein of H. influenza (HI1604) and the glvr-1 deduced protein of M. leprae (MiPit). (*) and (.) indicates amino acid identity and conserved, respectively.
Fig. 4-6b: CLUSTAL W alignment between the pit deduced protein of *R. meliloti* (RmPit), the pitA deduced protein of *E. coli* (EcPitA), the pho-4*+* deduced protein of *N. crassa* (NcPho4) and the HI1604 deduced protein of *H. influenza* (HI1604). (★) and (.) indicates amino acid identity and conserved, respectively.
Fig. 4-6c: Pairwise alignments between the pit nucleotide sequences of *R. meliloti* (nRmpit) and *M. leprae* (nMlpit). (★) indicates identical nucleotides.
the protein encodes a membrane protein. Nine "certain" membrane-spanning domains were determined by the TopPred II program and a model for the secondary structure of the deduced protein is presented in Fig. 4-7. If RmPit acts as a secondary transporter with a 6+6 transmembrane helices construction (Nikaido and Saier, 1992) as Ncpho-4+ and EcPitA are suggested to be (Mann et al., 1989; Wanner, 1996), an active RmPit protein may require the formation of a dimer.

b) The OrfA protein

A GenBank search revealed that the OrfA protein (214 aa) was similar to the deduced protein of Haemophilus influenza HI1603 open reading frame (226 aa). Alignment of these proteins revealed 21% amino acid identity and 39% conserved (Fig. 4-8a). This result was particularly interesting as this gene was located just upstream from the pit-like HI1604 gene of H. influenza. Protein HI1604 shared 27% amino acid identity with the Pit protein (see above). In addition, the HI1603 and HI1604 genes were separated by 25bp and thus probably transcribe on a single transcript as appeared to be the case with the orfAl/pit genes of R. meliloti. No orfA-like open reading frame was detected upstream from mlglvr-1 and EcpitA and, except for HI1603, no other orfA-like gene was detected in GenBank searches.
Fig. 4-7: Top; Hydropathy plot generated by the Tmpred program (Hofmann and Stoffel, 1993). Bottom; Drawing created by the TopPred II program (Claros and von Heijne, 1994) representing the secondary structure for the pit deduced protein. KR represent the number of Lys and Arg; LL, the loop length and KR Diff, the positive charge difference.
Fig. 4-8: Top (a); Pairwise alignment between the orfA deduced protein of *R. meliloti* (OrfA) and the HI1603 deduced protein of *H. influenza* (HI1603). (•) and (••) indicates amino acid identity and conserved, respectively.

Bottom (b); OrfA protein sequence showing the potential secretory signal (boxed) and a putative transmembrane domain (underlined).
Analysis of the deduced-protein sequence of orfA predicted a secretory signal sequence with a potential cleavage site between position 28 and 29 (Fig.4-8b) which conformed to the (-3, -1) rule (Oliver, 1985), suggesting that the protein may be periplasmic. A search for a potential membrane spanning domains using the Tmpred method suggested the presence of a putative transmembrane helix from amino acid 116 to 135 implying that the protein may be membrane bound. The HI1603 deduced protein did not show any potential secretory signal sequence in its N-terminal region, nor did it possess any potential membrane spanning domain. This suggests that secondary structure and cell localization of these two proteins may be different and thus may have distinct functions.

c) The RecF and Orf2 proteins

GenBank searches with the DNA upstream of orfA revealed a partial open reading frame which was homologous to many RecF proteins In E. coli, RecF is required for the resumption of the replication at DNA replication forks (Courcelle et al., 1997; Kogoma, 1997). The highest homology was with the RecF protein of Caulobacter crescentus (388aa; Rizzo et al., 1993), where the deduced 176 amino acids of the R. meliloti RecF-like protein shared 43.2% identity; 57.4% conserved with the C. crescentus RecF protein (Fig 4-9). The homology
Fig. 4-9: Pairwise alignment between the recF deduced protein of *R. meliloti* (RmRecF) and the recF deduced protein of *C. crescentus* (CcRecF). (•) and (•) indicates amino acid identity and conserved, respectively.
extended over the N-terminus of RecF and hence this gene is transcribed divergently from the orfA pit genes.

In GenBank searches with the partial orf2 region, located downstream from pit, we failed to detect orf2-like genes.

5- **Subcloning of the sfx1 wild type locus**

To investigate the nature of the sfx1 mutation, we cloned the orfA pit region from the wild type strain. As the 4.8kb HindIII-Sacl fragment contained the entire sfx1 locus (see above) and was present in the wild type strain (see Fig. 4-1b), Rm1021 (wild type) genomic DNA was digested with the HindIII and Sacl restriction enzymes. The resulting fragments were subcloned into the pUC118 vector digested with the same enzymes. *E. coli* DH5α competent cells were transformed with the religated DNA and the 2000 to 3000 Ap r transformants obtained per plate were screened by colony hybridization using pTH276, the 4.8kb HindIII-Sacl fragment of pTH90, as probe. Plasmid DNA from two clones which hybridized to the probe were further examined by restriction with the EcoRI, Sacl, HindIII and Sacl/HindIII enzymes. Only clone #1 carried a 4.8kb HindIII-Sacl fragment similar to the one found in pTH90 (data not shown). This clone, designated pTH354, contains the wild type sfx1 locus.
6- Localization of the sfx1 mutation

To determine whether the sfx1 mutation lay in the orfA-recF intergenic region (Fig. 4-4a), XhoI/EcoRI (3) and EcoRV(1)/EcoRI (3) fragments from the sfx1 locus were cloned, together with a Nm rib marker, into pBR322 to give pTH396 and pTH397 respectively (see Material and Methods). The two plasmids were transferred into RmG490 (phoCΩ490) and the Nm rib recombinants arising via a Campbell-type single crossover in the RmG490 genome were isolated. These colonies were screened on GYM medium, where phoCDET mutants generate a mucoid colony phenotype as opposed to the dry colony phenotype of the wild type and phoCΩ490 sfx1 strains. Suppression of the phoCΩ490 mucoid phenotype in these recombinants was dependent on: (i) the presence and location of the sfx1 mutation in the cloned fragment and (ii) the location of the recombination event. 12 out of the 70 (17%) pTH396 recombinants formed dry colonies on GYM medium and thus suppressed the mucoid phenotype of RmG490. Also, these twelve recombinants grew similarly to the wild type in MOPS medium containing 2mM phosphate (data not shown). All 70 pTH397 recombinants tested formed mucoid colonies on GYM. Together these data indicate that the sfx1 mutation lay between the XhoI and EcoRV(1) restriction sites of pTH276 (orfA-recF intergenic region).

The XhoI/EcoRV(1) fragments from the sfx1 locus in pTH380 and the wild type locus in pTH354 were subcloned in pUC118. The DNA sequence of each
**Fig. 4-10:** Pairwise alignment of the wild type and sfx1 XhoI-EcoRV sequences. (★) indicates nucleotide identity. Only one mismatch is observed; deletion of a thymidine residue in the hepta-thymidine region of the sfx1 sequence.
fragment was determined. Both sequences were identical, except for a missing T in a hepta-thymidine region of the wild type sequence (i.e.: 7Ts in wild type sequence versus 6Ts in the sequence from the sfx1 locus; Fig. 4-10). This region is centered 81 residues upstream from the orfA start codon.

In summary, these data confirmed that the sfx1 mutation is located within the Xhol/EcoRV(1) fragment of pTH276 and provide strong evidence that the thymidine deletion in the sfx1 sequence is responsible for the phenotypic differences between the wild type and the sfx1 orfA pit locus.

7- Effect of sfx1 on orfA and pit expression

To investigate whether the genotypic difference between the sfx1 and wild type locus resulted in differences in orfA pit expression, we constructed transcriptional lacZ fusions to orfA and pit from both the wild type and sfx1 locus. The fusions, which included the recF-orfA intergenic region, were made by subcloning the 2.1kb EcoRI and the 1kb EcoRI/SphI fragments of pTH380 (sfx1) and pTH354 (wild type) into the broad host range plasmid pMP220 (see Materials and Methods). In addition, chromosomal pit fusions in both wild type and sfx1 strains were constructed in which a cassette (lacZmobSp) was inserted immediately following the pit gene such that lacZ was transcriptionally fused to pit (see Materials and Methods).
Fig. 4-11: Histogram showing the alkaline phosphatase (a) and β-galactosidase activities (b) of the *pit* and *orfA::*lacZ plasmid fusions containing strains after 38 hours growth in MOPS-buffered minimal media with no phosphate added (■) and supplemented with 2mM phosphate (□). The plasmid fusions were made by subcloning the *pit* and *orfA* genes from both wild type (wt) and *sfx1* loci in pMP220. Each data point represents the average of triplicate values ± S.E. (error bars).
Fig. 4-12: Growth (a), alkaline phosphatase (b) and β-galactosidase activities (c) of pit::lacZ chromosomal fusions from wild type and sfx1 strains after 22, 38, 44 and 62 hours growth. (a); ○, ◊: pit fusion from a wild type background; ▲, Δ: pit fusion from a sfx1 background. ○, ▲: cells grown in MOPS P0; ◊, Δ: cells grown in MOPS P2. (b and c); pit chromosomal fusions from wild type (wt) or sfx1 background strains grown in MOPS P0 or P2 media. The β-galactosidase activities presented are corrected from the activity obtained when the lacZ gene was fused in the opposite orientation relative to pit. This activity was about 10 units whatever the growth conditions. Each data point represents the average of triplicate values ± S.E. (error bars).
Expression of the wild type and \textit{sfx1 orfA pit} fusions were tested after 38 hours growth in MOPS-buffered media with no phosphate (P0) or 2mM phosphate (P2) added. High and low alkaline phosphatase (AP) activities confirmed that the cells were grown under phosphate deficient and phosphate sufficient conditions respectively (Fig. 4-11a and 4-12b). The expression of \textit{sfx1-pit}, as measured by the level of \(\beta\)-galactosidase activity of plasmid-borne fusions, was 3 and 5 times higher than the wild type-\textit{pit} fusion following growth in MOPS P0 and MOPS P2, respectively (Fig. 4-11b). Similarly, expression of the \textit{sfx1-orfA} fusion was about 3-fold higher than the wild type-\textit{orfA} fusion. Higher levels of expression for all the plasmid born fusions were obtained when the cells were grown in MOPS P2 as opposed to MOPS P0 suggesting that \textit{orfA pit} expression increased when the cells were grown under phosphate sufficient conditions. In all backgrounds and whatever the growth condition of the cells containing the fusions, the level of \textit{orfA} expression was 3- to 5-fold higher than the level of \textit{pit} expression.

The chromosomal fusions showed lower levels of expression than the plasmid fusions as expected due to the lower copy number of the genes. (As in the case of the plasmid fusions, the chromosomal \textit{pit} fusions showed 3- to 5-fold more expression in the \textit{sfx1} than in the wild type background (Fig. 4-12c). Analyzing the expression of the fusions at various stages of growth revealed a higher level of \textit{pit} expression in the exponential phase of growth (22 hours).
When reaching the saturation phase of growth, *pit* expression decreased to a constitutive low level of expression that remained more than 3-fold higher in the *sfx1* than in the wild type background. Thus, consistent with the results of the plasmid fusions, the *sfx1* mutation led to at least 3-fold increase in *pit* expression. Phosphate regulation was observed, after 22 hours growth (exponential phase), in both the wild type and *sfx1* background with 3 times more expression for the cells grown in MOPS P2 than in MOPS P0. This regulation was however not observed in the wild type background and was gradually reduced in the *sfx1* background as the cells settled in saturation phase suggesting a growth phase regulation of the locus. This apparent lack of phosphate regulation in the wild type background may reflect the very low level of expression (limit of detection) measured from these fusions.

In summary, our results show increased *orfA pit* expression due to the *sfx1* mutation. The *orfA pit* expression appears to be under the influence of the phosphate content of the media with increased expression of *orfA pit* in response to high external phosphate concentration. This phosphate-induced regulation was the reverse of what was observed for the genes of the Pho regulon.

8- **Effect of the *phoCQ490* mutation on *orfA* and *pit* expression**

Using plasmid-borne fusions, we also investigated whether the *phoCDET* mutation affected *orfA pit* expression. The plasmids from both wild type and *sfx1*
locus were mated into RmH667 (Lac⁺; phoCΩ490) and assayed for β-
galactosidase expression after 38 hours growth in MOPS P0 or MOPS P2. The
alkaline phosphatase activity was as expected; with the phoCDET derived
strains showing high alkaline phosphatase activity when the cells were grown in
MOPS P0 and MOPS P2 (Fig. 4-13a; see also Bardin et al., 1996, Fig. 5). The
phoCΩ490 mutation reduced the expression of orfA pit from a wild type promoter
so that the level of expression of the fusions was the same whether they were
from MOPS P0 or MOPS P2 grown cells (Fig. 4-13b). An identical result was
obtained when the phoCΩ490 mutation was transduced in the chromosomal
pit::lacZ fusion strain (Fig. 4-13d). This provides additional evidence that
phoCDET cells behave as if they were phosphate starved. The level of sfx1-
pit::lacZ chromosomal fusion in a phoCΩ490 background was identical to the
level of expression in a wild type background. This result, together with the lack
of AP activity from MOPS P2 grown cells (Fig. 4-13c), confirmed that the sfx1-
orfA pit is functional in these constructs. Expression of the sfx1-orfA and sfx1-pit
plasmid fusions from MOPS P2 grown cells was reduced by 23 to 30% in a
phoCDET background. However, their level of expression was still 2 to 3 times
higher than the level of the wild type fusions in a wild type background grown in
MOPS P2. A 2- to 3-fold increase in orfA pit expression, due to the sfx1
mutation, appeared here again to be sufficient to allow suppression of the
phoCDET phenotypes.
Fig. 4-13: Histogram showing the alkaline phosphatase (a) and β-galactosidase activities (b) of the pit and orfA::lacZ plasmid fusions containing strains after 38 hours growth in MOPS-buffered minimal media with no phosphate added (■) and supplemented with 2mM phosphate (□). The plasmid fusions, made by subcloning the pit and orfA genes (Gene fusion) from both wild type (wt) and sfx1 loci (Plasmid locus), were mated in both wild type (wt) and H667 (phoCΩ490) backgrounds. Each data point represents the average of triplicate values ± S.E. (error bars).
Fig. 4-13: Histogram showing the alkaline phosphatase (c) and β-galactosidase activities (d) of the wild type- and sfx1-pit::lacZ chromosomal fusions after 32 hours growth in MOPS-buffered minimal media with no phosphate added (■) and supplemented with 2mM phosphate (□). The fusions were tested in both wild type and phoCΩ490 backgrounds. The β-galactosidase activities presented are corrected from the activity obtained when the lacZ gene was fused in the opposite orientation relative to pit. This activity was about 10 units whatever the growth conditions. Each data point represents the average of triplicate values ± S.E. (error bars).
It is interesting that sfx1-derived fusions were still phosphate regulated in the phoCΩ490 background. As these cells are phosphate starved even when they are grown in MOPS P2 media, as indicated by the high level of AP measured, we would have expected sfx1-pit and sfx1-orfA expression to be the same in both MOPS P0 and MOPS P2 phoC grown cells. High expression of the fusions in MOPS P2 suggested that the sfx1 promoter mutation enabled the locus to be induced by the high external phosphate concentration probably by enhancing the binding of a phosphate-dependent transcriptional activator. The mechanism by which this occurred and the protein(s) involved in this regulation remain to be characterized.

9- Increasing the wild type orfA pit copy number suppresses the phoCΩ490 growth phenotype

The data obtained from the above expression studies suggested that a small increase (3x) in orfA pit expression was sufficient to suppress the phenotypes associated with the phoCDET mutation. We therefore cloned the wild type orfA pit locus as a partial 2.6kb EcoRI fragment from pTH354 into the broad host range vector pRK7813. The resulting plasmid, pTH391 was mated into RmG490 (phoCΩ490). As a control experiment, we also mated plasmid pTH348, carrying the sfx1 region, into RmG490. Both of these transconjugants grew like the wild type in MOPS media containing 2mM phosphate (Fig. 4-14).
Fig. 4-14: Growth of Rm1021 (wt, ■), RmG490 (phoCΩ490, ●), RmH607 (phoCΩ490 / pTH348, ◊) and RmH841 (phoCΩ490 / pTH391, □) in MOPS-buffered minimal media supplemented with 2mM Pi. Each time point represents the average of triplicate values.
This observation confirmed that increasing the copy number of the wild type orfA pit locus was sufficient to allow wild type growth of the \textit{phoCΩ490} mutant in media containing 2mM phosphate.

10- Role of orfA pit in divalent cation assimilation

Van Veen et al. (1994a and b; 1993b) showed that a metal-phosphate (\textit{MeHPO}_4, with \textit{Me} being either \textit{Ca}^{2+}, \textit{Mg}^{2+}, \textit{Mn}^{2+} or \textit{Co}^{2+}) but not Pi was translocated via the Pit system in \textit{E. coli} and \textit{A. johnsonii}. Preliminary experiments were performed to address whether the orfA pit locus of \textit{R. meliloti}, characterized in this study, was influenced by the \textit{Ca}^{2+} and/or \textit{Mg}^{2+} divalent cations present in the MOPS-buffered minimal medium. Growth of the wild type strain Rm1021, RmG490 (\textit{phoCΩ490}), RmG762 (\textit{phoCΩ490, sfx1}) and RmG591 (\textit{sfx1}) was examined in MOPS P2 media in the presence or absence of either \textit{Ca}^{2+}, \textit{Mg}^{2+} or both of these cations (Fig. 4-15). The absence of \textit{Mg}^{2+} dramatically reduced the final growth yield of all cultures (\textit{OD}_{600} of 0.3 at 0mM \textit{Mg}^{2+} vs \textit{OD}_{600} of 1.0 in 2mM \textit{Mg}^{2+}). The yield was even more reduced when both \textit{Ca}^{2+} and \textit{Mg}^{2+} were withdrawn from the media (reaching an \textit{OD}_{600} of 0.25 for the wild type strain). The absence of either \textit{Ca}^{2+} or \textit{Mg}^{2+} from the media did not result in any growth phenotype of the \textit{sfx1} containing strains. However, in cultures lacking both \textit{Ca}^{2+} and \textit{Mg}^{2+} the yield of RmG591 and RmG762 cultures was reduced when compared to the wild type strain. Strain RmG762 in which phosphate
Fig. 4-15: Growth of Rm1021 (wt, ■), RmG490 (phoCΩ490, ●), RmG762 (phoCΩ490, sfx1, ★) and RmG591 (sfx1, ▲) in MOPS-buffered minimal media supplemented with 2mM Pi (MOPS P2) and lacking CaCl₂ (MOPS P2 -Ca), MgSO₄ (MOPS P2 -Mg) or lacking both divalent cations (CaCl₂ and MgSO₄; MOPS P2 -Ca -Mg). Each time point represents the average of triplicate values.
uptake appears to occur primarily via the Pit system still grew significantly more than RmG490 (phoCΩ490), suggesting that even though the Pit system may preferably translocate metal phosphate, it is able to take up ortho-phosphate as well. The levels of pit expression in wild type or sfx7 backgrounds, as determined by the level of β-galactosidase expression from lacZ plasmid fusions, was not affected by the absence of divalent cations in the growth media (data not shown).

Our experiments demonstrated that Mg\(^{2+}\) cations are required for growth. *R. meliloti* strains, dependent on the Pit transport system for phosphate uptake, show a slight growth reduction in media lacking both Ca\(^{2+}\) and Mg\(^{2+}\) divalent cations. We note however that further experiments will have to be done in order to directly address the substrate specificity of the Pit system.
D. Discussion

The sfx1 locus of *R. meliloti* was previously identified through the genetic analysis of second-site mutations which suppress the symbiotic Fix′ phenotype of ndvF mutants (Oresnik et al., 1994). The *ndvF* locus contains four genes, *phoCDET*, which encode a phosphate transport system (Chapter III; Bardin et al., 1996).

This chapter reports a genetic characterization of the sfx1 suppressor mutation. The locus, localized in a 2kb EcoRI-EcoRV fragment, contains two open reading frames designated orfA and pit. Because the two genes partially overlap and showed the same type of regulation, they probably constitute an operon in the order, orfA-pit. The predicted *R. meliloti* Pit protein is homologous to phosphate transport proteins previously identified in *E. coli* and *Neurospora crassa* (Sofia et al., 1994, Mann et al., 1989). Suppression of the symbiotic and growth phenotypes associated with mutation in the *ndvF* (*phoCDET*) locus by *sfx1* suggests that the loss of one phosphate transport system (*phoCDET*) was rescued by mutation in a second phosphate transporter (*orfA pit*). Evidence that *sfx1* was dependent upon a functional *orfA-pit* locus included (1) Tn5 mutations in *pit* and/or *orfA* abolished suppression of both the symbiotic and growth phenotype of a *phoCDET* strain and (2) Pi uptake of a *phoCDET* mutant strain
could not be restored when the pit gene was interrupted by a Tn5 insertion (Dr. Voegele, personal communication).

In the absence of the major phosphate transport system encoded by *phoCDET*, the wild type orfA pit allele was not able to assimilate enough phosphate to sustain the metabolic functions of the cell (even when the cells were grown in a media containing high phosphate concentration). This raises questions concerning the regulation of expression of this locus.

The work presented here strongly suggests that the low level of orfA pit expression is responsible for the lack of phosphate assimilation in the *phoCDET* mutant strains. Employing chromosomal and plasmid-borne transcriptional lacZ gene fusions, we found that the orfA pit expression from the sfx1 locus was 3 to 5 times higher than orfA pit expression from the wild type locus. In addition, increasing the expression of the wild type orfA pit locus by subcloning it in a multicopy plasmid (like pRK7813) was sufficient to suppress the growth phenotype of a *phoCDET* mutant. Preliminary results also indicated that this plasmid restored some Pi uptake of *phoC\(\Omega\)490 strain (data not shown). Suppression by the wild type orfA pit locus cloned on a low copy number plasmid vector (pLAFR1, that contained 2 to 4 copies per cell; data not shown), as well as the 3- to 5-fold increase in expression of the locus by the sfx1 mutation suggests that as little as 3 extra copies of the wild type locus may be sufficient to assimilate enough phosphate to support normal cell growth for *phoCDET*
mutants in MOPS P2 and to fix nitrogen efficiently when inoculated on alfalfa plants.

orfA pit expression, whether in a wild type or sfx1 background is also under phosphate regulation. Higher levels of expression were measured when the cells containing the orfA and pit::lacZ plasmid-borne fusions were grown under phosphate sufficient conditions (2mM Pi) than phosphate limiting conditions (0mM Pi). Similar results were obtained when exponentially growing cells containing the pit chromosomal fusions in both the wild type and sfx1 backgrounds were examined. orfA pit is then phosphate regulated in an opposite manner than the genes of the Pho regulon. Pit may then be the main Pi transporter under phosphate sufficient conditions while phoCDET would take up phosphate mainly when Pi concentrations in the environment are reduced. The inability of the orfA pit wild type locus to assimilate enough phosphate, under Pi sufficient conditions, to allow growth of a phoCDET mutant can be explained by the repression of the locus in these phosphate-starved cells. This suggested that pit expression may depend upon the internal, rather than external Pi concentration. Alternatively, the PhoCDET transport system may be involved in sensing the environmental Pi concentration and thus be indirectly involved in regulating pit expression. The involvement of PstSCAB, the high affinity phosphate transport system in E. coli, in sensing environmental phosphate has also been suggested (Wanner, 1996). It is interesting the locus directed by a
*sfx1* mutation was still expressed and was phosphate regulated under these conditions.

The biochemical characteristics of the Pit transport system are currently being examined in our laboratory by Dr. Voegele. He has confirmed that *pit* encodes a low-affinity phosphate transport system that constitutes the main phosphate transporter in a phosphate sufficient environment. On this basis, RmPit is similar to the PitA system of *E. coli*; Muda et al. (1992) postulated that Pit is the major phosphate transporter under high phosphate conditions, with Pst, the high-affinity phosphate transport system in *E. coli*, participating to only 30% of the Pi uptake. RmPit however differs from EcPitA as far as its regulation is concerned; EcPitA was shown to be constitutively expressed in *E. coli* (Willsky and Malamy, 1980a) while we presented evidence that it is phosphate regulated in *R. meliloti*.

The *sfx1* suppressor mutation was located in the promoter region of the *orfA* gene and was characterized as a deletion of a thymidine residue in an hepta-thymidine sequence located about 80 nucleotides upstream from the start codon of *orfA*. As this deletion appears to increase expression of the *orfA-pit* locus, the point mutation likely affected the binding site or destabilized a repressor component of the system. Alternatively, it may increase the stability of a phosphate-dependent activator/σ-factor/RNA polymerase complex to the promoter region leading to an increased level in *orfA pit* expression. In this
respect, Scholten and Tommassen (1993) showed that the level of \textit{phoE} expression could be increased in cells grown under phosphate sufficient conditions by modifying the Priibnow box of the promoter toward a more optimal consensus sequence.

Analysis of the promoter region revealed the presence of a putative Pho Box just upstream from the thymidine deletion suggesting that PhoB may regulate the locus. In \textit{E. coli}, PhoB transcriptionally activates the genes of the Pho regulon under phosphate limiting conditions (Wanner, 1996). As \textit{R. meliloti orfA pit} expression is repressed under these conditions, PhoB or a PhoB-dependent protein appears to negatively regulate the locus. PhoB has never been shown to act as a negative regulator in \textit{E. coli}, although Smith and Payne (1992) postulated that PhoB may negatively regulate, in response to a low phosphate environment, the expression of the periplasmic binding proteins involved in peptide transport in \textit{E. coli}. Analysis of \textit{phoB} mutants of \textit{Rhizobium meliloti} revealed its role as repressor of the \textit{orfA-pit} locus (see Chapter V). The \textit{sfx1} mutation, however, did not affect the PhoB repression as \textit{phoB} mutations affected \textit{orfA pit} expression in a similar manner whether fusions from the wild type or \textit{sfx1} locus were considered. The mechanism by which the \textit{sfx1} point mutation in the promoter region of \textit{orfA pit} leads to increased expression of the locus as well as the proteins involved in this induction are still unknown.
The role and function of orfA is unclear. The gene shows no homology with the GenBank database except with the HI1603 gene of Haemophilus influenzae (Fleischmann et al., 1995). HI1603 is itself located upstream from a pit-like gene in this organism. However it appears that these two proteins are structurally different and hence may have distinct functions. Because of its weak (if any) association with the membrane, it is unlikely that OrfA is involved in the transport itself and may rather be involved in regulating pit expression.

The PitA transporter in E. coli as well as the low affinity phosphate transport system of A. johnsonii have been shown to transport divalent metal-phosphate instead of Pi (Van Veen et al., 1994b; Van Veen et al., 1993b). Experiments in which the divalent cations were withdrawn from the growth media reduced the growth of cells depending on the Pit system for phosphate assimilation. The Pit system of R. meliloti may then have a similar substrate specificity to the Pit systems of A. johnsonii and E. coli (Van Veen et al., 1993b; Van Veen et al., 1994b). Additional experiments are however required to confirmed this observation.

In summary, suppression of the symbiotic phenotype of the phoCDET mutants occurred by increasing the level of expression of a locus, orfA pit, likely to encode a second phosphate transport system in R. meliloti. This provides additional evidence that it was the failure of phoCDET to take up phosphate that was responsible for the symbiotic phenotype of this strain. The mechanism by
which sfx1 increases orfA pit expression remains unclear. However, the point mutation (deletion of a thymidine in the hepta-thymidine region of what is believed to be the orfA pit promoter region) seems to be specific for suppression of the phenotypes associated with phoCDET mutations. It was indeed found that sfx4 and sfx5, other Class I suppressor mutations, isolated from independent inoculation experiments, possess the same deletion (N. Falcioni, personal communication).
Appendix B

- **Appendix B-1**: Restriction map of pTH61. pp. 164
pTH61 is a pUC18 derived plasmid containing the 12kb HindIII fragment of pTH90 (sfx1 locus). In addition to the global restriction map, the sites for a given restriction enzyme are shown on individual maps and the distances between the sites are indicated (in kb). BgIII (Bg); EcoRI (R), EcoRV (V), SacI (Sc), SmaI (Sm) and XhoI (X).

- **Appendix B-2**: Strategy of sequencing of the sfx1 locus. pp. 165
Drawing showing the overlapping fragments used to determine the 2828bp sequence containing the sfx1 mutation. The sequence is presented from the EcoRI (3) (see Fig. 4-4a) restriction site (nucleotide 1) to the Tn5 insertion Ω2-2 (nucleotide 2828).

- **Appendix B-3**: Sequence of the sfx1 locus. pp. 166-170
Nucleotide sequence from the EcoRI (3) (see Fig. 4-4a) restriction site (1) to the Tn5 insertion Ω2-2 (2828). The deduced amino acid sequence of each open reading frame is indicated and the arrow indicates the direction of translation. Also indicated are the location of the following restriction enzymes (over-line): BgIII (Bg); CiaI (C), EcoRI (ER), EcoRV (V), SacII (ScII), SmaI (Sm), Sphi (Sp) and XhoI (X).
Appendix B-1: Restriction map of pTH61

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Appendix B-2: Strategy of sequencing of the sfx1 locus
Appendix B-3: Sequence of the sfx1 locus

ER3

CTTAAGGCTCTTTCGGCAACGCAGCGTAGTCCGCGCCAGCTTGAGCGACCAGCCGCCG
AsnArgSerPheGlyThrAlaSerArgMetAlaArgAspPheGluSerAlaArgArg

70  80  90 100 110 120

GGCACAAAGCCCTAGCTCGCTTCTCTGGGCAAGGCTTCCTTCGGCCAGCCAGCCAGCCG
GlyHisGluProAspLeuSerLeuValLeuArgAspLeuPheArgArgArgAspAlaSer

130 140 150 160 170 180

CCCCGCACTCTCTCGGTAGGGTAGCAGGGACTCTCGGTGCGGATCTCACTAGGCAC
ProGlyThrPheLeuGlyAspMetAlaProThrLeuTrpLeuValLeuArgAlaHisAspThr

190 200 210 220 230 240

TCAAGCAGCAGCTGCGACAGGCGCGCTACGCGCAGCTGCGGCTAGGGTAGTAGCACTAAGGGGAGG
LeuGluAspValThrArgAlaAlaThrGlyAsnIleArgLeuArgArgSerGlnGlyGlu

250 260 270 280 290 300

AGCCAGGGAACGTCAAGGGCAGCTGAGGTGGCTGGGAAACGTACGCGAGCTGCGGCCGC
GluThrGlyGlnThrGlyThrGlyIleGluValSerGlyGluMetGlyAspValAlaAla

310 320 330 340 350 360

TTTGGCTCTTGGGTAGGCCCCCGCGGCTGGGCTGAGGTCTGCAACTAAGAAACGGCCG
PheValSerPheGlyAspProAlaGlyValArgAlaValAspAlaTyrAlaAlaArg

370 380 390 400 410 420

Sm

TTTGGCGCGGGCCCTTTTCTCTGCGGGGAGGTAGTCTAAAGAACGGCCG
LeuGlyArgGlyProSerLeuPheSerValGlyGluMetLeuAsnThrLysGlyAlaGly

430 440 450 460 470 480

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AsnGluGlyThrLeuValValHisArgGlnAspLeuAspLeuAlaLeuThrAlaTyrAsn
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IleThrTrpAsnIleValThrTrpValPheGlyIleProSerSerSerSerHisAlaLeu
1750 1760 1770 1780 1790 1800
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IleGlyGlyLeuValGlyAlaGlyLeuAlaLysThrGlyPheSerSerIleValTrpGln
1810 1820 1830 1840 1850 1860
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GlyLeuLeuLysThrAlaGlyAlaIleValMETSerProGlyIleGlyPheValLeuAla
1870 1880 1890 1900 1910 1920
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LeuLeuValLeuValLeuIleValSerTrpLeuPheValArgGlnThrProPheAlaValAsp
AGCACCTTCCGGGTCTGCAATTGCTTGCTCCCTCTCTATGCTCGCTGCGCATGGCGGC
SerThrPheArgValLeuGlnPheValSerAlaSerLeuTyrSerLeuGlyHisGlyGly
1990 2000 2010 2020 2030 2040
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AsnAspAlaGlnLysThrMETGlyIleIleAlaValLeuPheSerGlnGlyTyrLeu
2050 2060 2070 2080 2090 2100
ER
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2110 2120 2130 2140 2150 2160
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2170 2180 2190 2200 2210 2220
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AsnProMETGlnGlyPheCysAlaGluThrGlyGlyAlaIleThrLeuPheAlaAlaThr
2230 2240 2250 2260 2270 2280
TGGCTCGGCAATTCCGGTTACCACACCACACATACTACCGGCGCATCATGGCGTCGCC
TrpLeuGlyIleProValSerThrThrHisThrIleThrGlyAlaIleIleGlyValGly
• **Appendix B-4:** Tentative to determinate the transcriptional start site upstream of orfA by primer extension. The primer extension experiment was performed as stated in Bardin et al. (1996) using wild type (Rm1021) and sfx1 (RmG591) RNA extracted from LB grown cells. The primer used is boxed in the sequence below with the 5' and 3' direction indicated. This experiment was not successful as many bands appeared on the autoradiograph and were very faint. The nucleotides at which the main bands appeared are indicated by \( \downarrow \). Also indicated are the orfA translational start codon (+1), downstream of a putative TATA box (bold), the hexa-thymidine sequence (from an sfx1 promoter, underlined) and the putative Pho Box (hatched over-line). The location of the EcoRV and Smal restriction sites are over-lined.

\[
\begin{align*}
\text{EcoRV} & \quad \downarrow \downarrow \\
\text{GATATCGACGTGCTGCTAGGATGAAAGCCCGGCA} & \quad -185 \\
\text{AGGCAAGGCGAAAAAGGTGTTCTGTCCGCCCTCTCCCACC} & \quad -145 \\
\text{CTGGCGCGATTTCCGTATCCCGGGCTGCGCATTTCCGCCCA} & \quad -105 \\
\text{ATCGCCTGTGGAAAGCGCTTTTTTCGGCGCATGGAAC} & \quad -65 \\
\text{TTTCCCCGAGCGCGAAGTACAATATGACAACGTGCCTAC} & \quad -25 \\
\text{AATCGACAGTGATGGATCGCTGATACTCGGCCCTTTC} & \quad +16 \\
\text{GCAAGCTCCTCCCCGGGAAAGACCGTTCTTGACCTCTT} & \quad +56 \\
\text{CGCCCAGATCATTCGCACCCTGCTAGGGGCGCGAGGCA} & \quad +96 \\
\end{align*}
\]

PRIMER
CHAPTER V

Characterization of the sfx2 locus

A- Introduction

N$_2$-fixing root nodules result from a complex interaction between bacteria of the family *Rhizobiaceae* and leguminous plants. We recently characterized the symbiotic *ndvF* locus of *R. meliloti* and showed that it consists of four genes *phoCDET* which encode an ABC-type phosphate transport system (Bardin et al., 1996). Strains carrying insertion mutations in any of the *phoCDET* genes grew poorly in media containing 2mM inorganic phosphate and formed nodules which contained few bacteria and failed to fix N$_2$ (Fix$^-$ phenotype). Occasionally, we observed pink Fix$^+$ root-nodules on the plants inoculated with *phoCDET* mutants. Genetic analysis of isolates from these nodules revealed that they carried second-site mutations which suppressed the *phoCDET* Fix$^-$ phenotype. Two suppressor classes (I and II) were distinguished according to phenotypic characteristics and their position on the *R. meliloti* chromosome (Oresnik et al., 1994). In chapter IV of this thesis, we described the subcloning and sequencing
of the class I suppressor locus carrying the sfx1 mutation. The sequence revealed the presence of two open reading frames, designated orfA and pit, that probably form an operon. The deduced pit protein is homologous to phosphate transport proteins, including the low-affinity phosphate transport protein (Pit) of E. coli (Wanner, 1996). The orfA gene shows similarity to an open reading frame in H. influenza which also lay upstream from a pit-like gene but no homologous gene was present upstream of the pit gene of E. coli. Expression of orfA-pit at the R. meliloti sfx1 locus was found to be at least three times higher than expression in a wild-type background. We hypothesized that the increased orfA-pit expression results in increased phosphate uptake via the Pit-like protein and hence suppresses the phosphate and symbiotic phenotypes associated with phoCDET mutations.

In this chapter we report that the Class II suppression mutation, sfx2, maps to the phoUB locus of R. meliloti. We show that phoUB insertion mutants suppress the symbiotic, growth and mucoid phenotypes of phoCΩ490 mutation. Suppression by these mutants appears to occur by derepression of the orfA-pit genes thus allowing increased Pi uptake via the Pit transport system of R. meliloti.
B- Results

1- Strains carrying the sfx2 mutation are deficient in alkaline phosphatase expression.

The levels of alkaline phosphatase (AP) activity detected in wild type cells cultured in MOPS-buffered minimal media with no added phosphate (MOPS P0) are ten to twenty fold higher than the AP activity found in cells grown in the same media containing 2mM inorganic phosphate (MOPS P2) (Fig. 5-1 and Bardin et al., 1996). Therefore, as is the case for many bacteria, measurements of alkaline phosphatase activity in *R. meliloti*, can be used to monitor the cell physiological status with respect to phosphate availability.

Experiments with the *R. meliloti* phoCΩ490 mutant, RmG490 (mutant representative of the different phoCDET mutants studied, see Chapter III), revealed a high AP activity when cultured either in the absence of added phosphate or in media containing 2mM Pi (Fig. 5-1). Thus even in the presence of 2mM Pi, RmG490 cells appeared to be starved for phosphate and this phenotype is consistent with the observation that RmG490 grows poorly in media containing 2mM Pi (Fig. 6; Bardin et al. 1996; see also Appendix A in Chapter III). As we had previously found that *R. meliloti* phoCDET mutants grew like the wild type in MOPS media containing 2mM aminoethylphosphonate (AEP) as a P source, we measured AP activity in cultures of RmG490 and the wild-type
Fig. 5-1: Alkaline phosphatase (AP) activity of Rm1021 (wt), RmG490 (phoC), sfx2 strains in phoC\(\Omega\)490 or wild type backgrounds (phoC/sfx2 or sfx2, respectively) and sfx1 strains in phoC\(\Omega\)490 or wild type backgrounds (phoC/sfx1 or sfx1, respectively) after 60 hours growth in MOPS-buffered minimal media with no phosphate added (MOPS P0, solid box) or containing 2mM inorganic phosphate (MOPS P2, open box). The AP activity of Rm1021 and RmG490 was also determined after 80 hours growth in MOPS-buffered minimal media containing 2mM aminoethylphosphonate (MOPS AEP, hatched box). Each activity represents the average of triplicate values ± S.E..
Rm1021 following growth in this medium (Fig. 5-1). Both RmG490 and wild-type strains contained low background AP activities after growth in 2mM AEP. Thus, unlike what is observed with *E. coli* where mutants defective in the high affinity transport system (*pstSCAB*) showed constitutive AP expression (Cox et al., 1989), AP expression in the *R. meliloti* *phoC*Ω490 mutant was repressed when a readily assimilated phosphorus source like AEP was provided in the growth media.

To examine the effect of the previously isolated *ndvF* (*phoCDET*) symbiotic suppressor mutations on alkaline phosphatase expression, the class I and class II suppressor mutations, *sfx1* and *sfx2* respectively, were introduced into a *phoC*Ω490 mutant to generate the strains RmG762 and RmH363, respectively. AP activity in these strains was repressed in the presence of 2mM Pi (Fig. 5-1). Unexpectedly however, no alkaline phosphatase activity was detected when RmH363 (*phoC sfx2*) was grown in MOPS medium with no Pi added (P0). This result was in contrast with what we observed with the wild type (wt), RmG591 (*sfx1*) and RmG762 (*phoC sfx1*) strains. Subsequent analysis revealed that RmG497 cells which carry the *sfx2* mutation in an otherwise wild-type background also lacked alkaline phosphatase activity (*AP*− phenotype) (Fig. 5-1, see also Appendix C-1). The other Class II suppressor, *sfx3*, in a *ΔphoCDET* background, also showed no AP activity when grown in MOPS P0 (see Appendix C-1). The *AP*− phenotype of strains carrying Class II mutation,
together with suppression of the mucoid colony and symbiotic phenotypes associated with the phoCDET (ndvF) mutations (Oresnik et al., 1994) suggested that sfx2/3 probably affects regulatory gene(s) involved in phosphorus assimilation similar to the phoBR genes of *E. coli* (see Wanner, 1993).

Using the AP\(^-\) phenotype of the sfx2/3 containing strains, we employed a screening method for identifying these mutants by plating the cells on LB media supplemented with 60\(\mu\)g/ml of 5-bromo-4-chloro-3-indolyl phosphate (X-Phos\(^{60}\)). As the sfx2/3 mutants were unable to hydrolyze the chromogenic compound the colonies remained white, while blue colonies were obtained with the wild type strain Rm1021.

2- **Mapping of the sfx2 locus**

Tn5 and Tn5-233 insertions linked to sfx2 were previously isolated as follows: lysates made from a pool of 6000 random Tn5 insertions (Bank NM1) and a pool of 2000 random Tn5-233 insertions (Bank GS2) were transduced into RmF346 (ΔphoCDET sfx2, deoxycholate sensitive; Oresnik *et al.*, 1994). IndividualNm\(^-\) and Gm\(^-\)-Sp\(^-\) transductants were screened for loss of sensitivity to deoxycholate. Three Tn5 insertions, designated RmG549 (Ω5256), RmG551 (Ω5258) and RmG552 (Ω5259) and three Tn5-233 insertions, designated RmG639 (Ω5262), RmG640 (Ω5263) and RmG641 (Ω5264) showed linkage to the deoxycholate sensitivity phenotype of the mutant strain. Here we determined
Fig. 5-2 Genetic linkage map showing the location of sfx2 relative to three Tn5 insertions (Ω5256, Ω5258 and Ω5259) and three Tn5-233 insertions (Ω5262, Ω5263 and Ω5264). The cotransduction frequency is represented in %.

Table 5.1: Two (a) and three (b) factor crosses used to calculate the linkage between the markers shown in Fig. 5-2. Linkages were determined by patching 50 transductant colonies on appropriate media. Linkages of Ω5262, Ω5263 and Ω5264 to Ω5258 are not reported in these tables as they were previously determined by L. Oresnik. Abbreviation used: (B): blue colonies on LBX-Phos<sup>60</sup>; (W): white colonies on LBX-Phos<sup>60</sup>; wt: wild type; G: Gm; S: Sp, (°) and (') meaning sensitive and resistant, respectively.
Table 5-1a: Two factor crosses

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% linkage:

Φ G549 → G640:  4 (B) /50 → 8% linked to sfx2
34 Gm^s-Sp^s /50 → 68% linked RmG640
Φ G552 → G640:  18 (B) /50 → 36% linked to sfx2
1 Gm^s-Sp^s /50 → 2% linked RmG640
the genetic linkage of these insertions to \textit{sfx2} using the alkaline phosphatase phenotype of the \textit{sfx2} mutation. The Nm\textsuperscript{r} or Gm\textsuperscript{r}-Sp\textsuperscript{r} of the insertions were transduced into the \textit{sfx2} strain RmG497 or the wild type strain Rm1021 and individual transductants were screened for the presence of blue or white colonies respectively on LBX-Phos\textsuperscript{60} plates. The linkages of these insertions to \textit{sfx2} and to one another is reported in Fig. 5-2 and Table 5-1a and b. Insertion \( \Omega 5258 \) was the closest to the \textit{sfx2} locus with a linkage of 66\% (about 21kb away from \textit{sfx2}, as determined from the Wu equation (Wu, 1966)). This linkage value was similar to the 70\% linkage value obtained when \( \Omega 5258 \) was transduced into RmF346 and the transductants were checked for resistance to deoxycholate (Oresnik et al., 1994).

The location of \textit{sfx2} on the \textit{R. meliloti} genome was determined using seven Tn5-\textit{mob} insertion strains as described in Oresnik et al. (1994). The Tn5-\textit{mob} insertions were transduced into strain RmG640 (\( \Omega 5263 \), 50\% linked to \textit{sfx2}; see Fig. 5-2). The highest frequency of Gm\textsuperscript{r}-Sp\textsuperscript{r} colonies was obtained from the strain transferring clockwise from \textit{trp-33}.

\textbf{3- Isolation of alkaline phosphatase deficient mutations linked to \textit{sfx2}.}

In an attempt to clone the wild type \textit{sfx2} locus, we transferred a wild type \textit{R. meliloti} clone bank into the \textit{sfx2} mutant RmG497 and screened the transconjugants for complementation of the \textit{AP}\textsuperscript{r} phenotype on LBX-Phos plates.
No blue colonies were obtained. As an alternate strategy, we sought to isolate additional sfx2-like mutations by screening pools of insertion mutants for AP⁻ colonies. A pool of random Tn5-132 (oxytetracycline resistant; Bank OT1) insertions in Rm1021 and a pool of random Tn5 insertions in Rm1021 (Bank NM1) were plated on LBX-Phos⁶⁰. One AP⁻ Tn5 insertion mutant (RmH405, pho27), and twenty six AP⁻ Tn5-132 mutants were identified and purified. Linkage of the Tn5-132 mutations to the sfx2 locus was examined by transducing Nm' from strain RmG551, carrying the Ω5258::Tn5 insertion (see Fig. 5-2), into these strains and screening for the presence of blue transductant colonies on X-Phos⁶⁰ plates. Three Tn5-132 insertion mutations, designated pho10, pho8 and pho3 (in which the Tn5-132 was replaced by Tn5-233), showed 60%, 64% and 66% linkage to Ω5258::Tn5 respectively (Table 5-2). These linkage values were similar to the 66% and 62% linkage obtained of sfx2 and sfx3 with Ω5258::Tn5, suggesting that the pho10, pho8 and pho3 insertions probably map to the sfx2-sfx3 locus. The other 23 white Tn5-132 insertion mutants showed no linkage to Ω5258::Tn5, however these were 100% linked in transduction to the Tn5 insertion in pho27 (strain RmH405). We also determined the linkage of pho10, pho8 and pho3 to Ω5259::Tn5 which maps on the opposite side of sfx2 (see Fig. 5-2). Nm' was transduced from strain RmG552 into the pho10, pho8, pho3, sfx2 and sfx3 recipient strains, and similar numbers of AP⁺
<table>
<thead>
<tr>
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<th>Recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain</td>
<td>insertion</td>
</tr>
<tr>
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<td>Ω5258:: Tn5</td>
</tr>
<tr>
<td>RmG552 (B)</td>
<td>Ω5259:: Tn5</td>
</tr>
<tr>
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<td>phoU10:: Tn5-233</td>
</tr>
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<tr>
<td>RmH852 (W)</td>
<td>phoB3:: Tn5-233</td>
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</tbody>
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Table 5-2: Linkage of sfx2, sfx3, phoU10, phoB8 and phoB3 to the Tn5 insertions Ω5258 and Ω5259. The linkage was determined by patching 50 Nm<sup>+</sup> transductants on LBX-Phos<sup>60</sup> and LBGm<sup>20</sup>-Sp<sup>100</sup> plates. For phoU10, phoB8 and phoB3, all the blue colonies on LBX-Phos<sup>60</sup> were also Gm<sup>+</sup>-Sp<sup>+</sup> while the white one were Gm<sup>-</sup>-Sp<sup>-</sup>, as expected. The linkage was determined by recording the number of blue (Gm<sup>-</sup>-Sp<sup>-</sup>) colonies. (B): blue colonies on LBX-Phos<sup>60</sup>; (W): white colonies on LBX-Phos<sup>60</sup>. 
recombinants were obtained in the five crosses (40 to 48%; Table 5-2). Together these data suggest that pho10, pho8, pho3 and sfx2/3 map close to each other at a locus between Ω5259 and Ω5258.

Following the transfer of the pLAFR1 wild type R. meliloti clone bank into the pho10 mutant (RmH399) we identified three cosmid clones (pTH282, pTH284 and pTH286) able to complement the AP⁻ phenotype of the mutant strain. Restriction analysis suggest that these clones shared common 20 and 24kb EcoRI as well as 10 and 26kb HindIII fragments. These cosmids, transferred into the pho8, pho3 and sfx3 mutants (RmH428, RmH430 and RmG425, respectively) also complemented the AP⁻ phenotype of these strains. When these plasmids were transferred into sfx2 mutants RmG497 and RmH318, no complementation of the AP⁻ phenotype was observed. This result together with the inability to isolate sfx2 complementing clone from the wild type Rm1021 clone bank (above) indicates that sfx2 may be a dominant mutation.

4- pho10, pho8 and pho3 map to the phoUB locus of R. meliloti

In order to identify the gene(s) in which the pho10, pho8 and pho3 insertions were located, we replaced the Tn5-132 insertions in these mutants with the TnV transposon. TnV possesses the gene encoding NptII (Km⁻) and the pSC101 origin of vegetative replication (able to replicate in E. coli but not in R. meliloti), flanked by the inverted repeats IS50_L and IS50_R (Furuichi et al., 1985).
The genomic DNA of these TnV containing strains was digested with SalI, religated and the ligation mixture was used to transform DH5α competent cells. As TnV does not possess any SalI restriction site, the religated plasmid will contain flanking genomic DNA. The Km· plasmids obtained this way were named pTH292, pTH311 and pTH287 for pho10, pho8 and pho3 insertions, respectively. Subclones of these plasmids were sequenced using the IS50 primer (5'- TCACATGGAAGTCAGATCCT -3'). Blast searches revealed that pho10 was located in the phoU gene and pho3 and pho8 in the phoB gene of R. meliloti (Fig. 5-3a and Appendix C-2). The DNA sequence of the R. meliloti phoB and part of phoU have been previously determined (accession number M96261; Cannon's group, unpublished data). The sequence revealed that these genes were adjacent to one another in the phoU-phoB order. The deduced PhoB protein was 47.6% identical (70% conserved) with the PhoB protein of E. coli, while the deduced C-terminal 117 amino acids from PhoU were 36% identical (52% conserved) with the corresponding region of the E. coli PhoU protein (see Fig 5-3b for alignments). The pho10 insertion was located 28 amino acids away from the end of the partial sequence of PhoU as determined from sequence alignment to the PhoU protein sequence of E. coli. Insertions pho3 and pho8 were located at position 157 aa and 188 aa of the R. meliloti PhoB sequence, respectively.
Fig. 5-3a  Map of the phoU-phoB locus of *R. meliloti* showing the location of the phoU10, phoB3 and phoB8 insertions. The complete sequence of phoB and partial sequence of phoU have been deposited in the Data Bank by Cannon’s group (accession number M96261; unpublished). The hatched part of the phoU gene represents the putative remaining portion of the gene determined from its alignment to the phoU gene of *E. coli*. A 276 nucleotide sequence of this portion has been sequenced in our laboratory (see Appendix C-2). The numbers above the figure correspond to the number of base pairs, while the amino acid numbers are indicated below the figure.
Fig.5-3b  Protein alignments between the PhoU and PhoB protein sequences of *R. meliloti* (pRm), deduced from the sequence deposited in the Data Bank, and the PhoU and PhoB protein sequences of *E. coli* (pEc). ★ and * indicates amino acid identity and conserved, respectively.
In view of these results the *pho10*, *pho8* and *pho3* alleles were designated *phoU10*, *phoB8* and *phoB3*, respectively. The linkage of sfx2/3 to *phoU10*, *phoB8* and *phoB3*, and the AP^- phenotype of these five mutants suggests that the *sfx2* and *sfx3* mutations are located in the *phoUB* locus of *R. meliloti*.

The Tn5-132 of pho27 was also replaced by a TnV transposon and the religated TnV containing plasmid was subcloned and sequenced. The sequence shows no significant homology with proteins deposited in the Data Bank.

5- *phoB* and *phoU* are required for growth on phosphonates.

The PhoB protein of *E. coli* is a transcriptional activator of the many genes expressed under phosphate deprivation, including the phosphonate uptake and degradation genes (Wackett et al., 1987). In order to investigate whether the *phoB*-like gene of *R. meliloti* also plays a central role in P assimilation, the ability of the *phoB* (and *phoU*) mutants to utilize various sources of phosphorus was determined and compared to the growth of the wild type, the *phoCΩ490* and *pho27* mutants. Growth experiments were performed in MOPS-buffered minimal media containing the phosphonates aminoethylphosphonate (AEP), ethylphosphonate (EP), aminomethylphosphonate (AMP) and methylphosphonate (MP) as well as the phosphorus compounds glycerol-3-phosphate (G3P), glucose-6-phosphate (Glc6P) and phosphoserine (PSer) at a
Fig. 5-4  Growth of sfx2, phoU and phoB mutants in MOPS-buffered minimal media with no phosphate added (MOPS P0), or containing 2mM of, inorganic phosphate (MOPS Pi), aminoethylphosphonate (MOPS AEP), ethylphosphonate (MOPS EP), aminomethylphosphonate (MOPS AMP), methylphosphonate (MOPS MP), glycerol-3-phosphate (MOPS G3P), glucose-6-phosphate (MOPS Glc6P) and phosphoserine (MOPS PSer). The growth of these mutants was compared to the growth of the wild type strain Rm1021, RmG490 (phoCΩ490) and pho27. The symbols are as follows: Rm1021 (wt, ■), RmG490 (phoCΩ490, ●), RmH610 (pho27::TnV, □) RmH836 (phoU10::TnV, ▼), RmH837 (phoB8::TnV, ◆), RmH838 (phoB3::TnV, ★) and RmG497 (sfx2, ▲). Each data point represents the average of triplicate values.
concentration of 2mM. Growth was compared with that obtained in phosphate-free media (MOPS P0) and in media containing 2mM Pi (MOPS Pi 2mM) (Fig. 5-4).

The sfx2, phoB and phoU mutants grew poorly on EP, MP and AMP indicating that the genes involved in the uptake and/or degradation of these compounds were under phosphate regulation and required functional PhoU and/or PhoB proteins for expression in R. meliloti. These growth phenotypes were clearly different to the one obtained using the phoCΩ490 mutant. The slow growth of phoCΩ490 was significantly higher than the growth of the phoUB mutants in EP while in MP and AMP phoCΩ490 grew to the level of the wild type with a delay of about 30 hours in MP. This suggested that the growth impairment of sfx2, phoB and phoU mutants in media containing EP, MP and AMP as sole phosphorus source was not due to lack of expression of the phoCDET phosphate transport system in these mutants (see below).

Following a lag of about 20-30 hours, both the phoU and phoB mutants grew at a growth rate comparable to the wild type in medium containing AEP. The growth lag in MOPS AEP suggests that an alternative system was induced in the phoUB mutants to assimilate this compound. In Enterobacter aerogenes (Lee et al., 1992) and Salmonella typhimurium (Jiang et al., 1995) the phosphonatase pathway that specifically assimilates and degrades AEP is Pho regulated and requires the PhoB protein for transcription. The system for uptake
and/or metabolism of AEP, induced in a phoUB mutant background in R. meliloti, was similar to the phosphonatase pathway as far as the specificity was concerned but did not require PhoB for expression. This may constitute a novel PhoB-independent pathway for utilization of AEP as phosphorus source.

Except for a slight delay of 4 to 6 hours, the phoUB mutants grew as well as the wild type on the organophosphates glycerol-3-phosphate (G3P), glucose-6-phosphate (Glc6P) and phosphoserine (PSer), suggesting that the assimilation and degradation of these compounds is unlikely to be Pho regulated (Fig. 5-4).

The growth of the mutant strains in MOPS medium containing 2mM Pi was similar to the growth of the wild type, although a slight growth delay (3 to 4 hours) was observed in the mutant cultures. In the absence of added phosphate, a small residual level of growth was usually observed in cultures inoculated with the wild type strain. We attribute this residual growth to the utilization of phosphate reserves such as polyphosphate. No residual growth was observed for any of the phoUB/sfx2 mutant strains, suggesting that a very limited phosphate reserve was present in these mutants. This correlates well with the finding that in Klebsiella aerogenes the ppk gene encoding the polyphosphate kinase enzyme which catalyzes polyphosphate formation is part of the Pho regulon (Kato et al., 1993) and that the E. coli ppk gene promoter possesses two putative Pho Boxes (Akiyama et al., 1992).
The *pho*27 mutation, disrupting an open reading frame showing no significant homology with protein of the Data Bank, showed no growth phenotype and the mutant grew as well as the wild type strain in the various phosphorus media.

We note that in all media, the *R. meliloti* *pho*U mutant had an identical phenotype to the *pho*B mutant. This differs considerably from the phenotypes of the *E. coli* *pho*U mutants that constitutively express the genes of the Pho regulon. A simple explanation for this result may lie in the fact that the *R. meliloti* *pho*U Tn5 insertion is likely to be polar on *pho*B (see discussion).

6- *pho*B is required for *pho*CDET expression

The induction (over 10 fold) of *pho*D:*lac*Z and *pho*E:*lac*Z gene fusions in response to phosphate starvation and the presence of two Pho-like Boxes in the promoter region upstream of the *pho*C gene suggested that *pho*CDET expression required a PhoB-like protein as transcriptional activator (Bardin et al., 1996). To investigate whether the *pho*B-like gene of *R. meliloti* characterized in this study was required for *pho*CDET expression, plasmid-borne *lac*Z fusions to *pho*D (insertion 7A) and *pho*E (insertion 19) (described in Bardin et al., 1996) were transferred into the Lac- *pho*B3 and *pho*B8 strains, RmH615 and RmH616 respectively. β-Galactosidase activity in these strains was measured after 38 hours growth in a MOPS-buffered minimal media with no phosphate added
Fig. 5-5 Alkaline phosphatase (a) and ß-galactosidase (b) activities of plasmid-borne ndvF::lacZ fusions in Lac⁺, wild type (wt), phoU10, phoB8 and phoB3 backgrounds after 38 hours growth in MOPS-buffered minimal media with no phosphate added (MOPS P0, solid box) or containing 2mM inorganic phosphate (MOPS P2, open box). The transcriptional lacZ fusions were due to Tn5-B20 insertions in the phoD (D7A) and phoE (E19) genes. Each data point represents the average of triplicate values ± S.E..
and media supplemented with 2mM Pi (MOPS P2). Alkaline phosphatase activity was also measured to verify the phosphate physiological state of the cells. As expected the Lac\(^-\) but otherwise wild type strain (wt) showed high AP activity when grown in MOPS P0 and low AP activity when grown in MOPS P2 while the phoB3, phoB8 and phoU10 derivatives showed low AP activity regardless of the growth medium (Fig. 5-5a). In the wild type background, both the phoD::lacZ and phoE::lacZ directed $\beta$-galactosidase activity was 4 to 5 times higher under P limiting conditions (MOPS P0) than P sufficient conditions (MOPS P2). In the phoB3 and phoB8 backgrounds $\beta$-galactosidase activity was not detected from either fusion even from cells grown in MOPS P0 (Fig 5-5b). The same phenotype was obtained when these fusion plasmids were mated into an sfx2 strain (strain RmH693; data not shown). In the phoU10 background (strain RmH617), phoD and phoE directed $\beta$-galactosidase activity was also dramatically repressed, with very little activity detected. It therefore appears that phoB (and perhaps phoU) are required for phoCDET expression.

7- **phoB and phoU mutations are able to suppress the growth, mucoidy and Fix\(^-\) phenotypes of phoCDET mutants.**

The phoU10, phoB8 and phoB3 mutations had phenotypic effects which were similar to the suppressor mutation sfx2. As the sfx2 mutation was originally identified as a suppressor of the Fix\(^-\) phenotype of phoCDET (ndvF) mutants we
<table>
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<th>Strain</th>
<th>Genotype</th>
<th>ARA ± S.E. (nmol/plant/hour)</th>
<th>% wt ARA</th>
<th>Dry Weight (mg/plant) ± S.E.</th>
<th>% wt Dry weight</th>
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<td>39.5 ± 2.7</td>
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<td>0</td>
<td>6.8 ± 0.6</td>
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**Table 5-3:** Symbiotic phenotype of sfx2, phoU10, phoB8 and phoB3 mutants in wild type and phoCΩ490 backgrounds. The acetylene reduction activity (ARA) was determined on 28 day old alfalfa root systems from three plants. Values are the mean of three independent measurements (total of nine roots) ± S.E.. The shoot dry weight represents the average of 3 times 10 plants ± S.E.. The percentages were calculated relative to the wild strain (Rm1021) values (set at 100%). UI: uninoculated.
tested the *phoB* and *phoU* insertion mutations for their ability to suppress the symbiotic as well as other phenotypes associated with the *phoCDE* mutations. The latter included the poor growth phenotype of *phoCDE* mutants in MOPS media containing 2mM Pi and their mucoidy colony morphology on low osmolarity media (GYM plates). The *phoUB-phoCΩ490* strains were constructed by transducing the *phoU10, phoB8* and *phoB3::TnV* (Nm') insertion alleles from strains RmH399, RmH428 and RmH430 into RmG490 (*phoCΩ490*) to create strains RmH623, RmH624 and RmH625, respectively.

Plants inoculated with the *ndvF* mutant *phoCΩ490* were small and chlorotic 28 days after inoculation and showed little evidence of N₂-fixation as measured by acetylene reduction and plant dry weight (Table 5-3). In the presence of *phoU10, phoB8* and *phoB3* mutations the Fix⁻ phenotype of the *phoCΩ490* mutant strain was suppressed to Fix⁺ with shoot dry weight and acetylene reduction values comparable to the wild type strain Rm1021 (Table 5-3). We also noted that the individual *phoB* and *phoU* mutants appeared to show no reduction in symbiotic effectiveness compared to the wild type strain.

In MOPS-buffered minimal media containing 2mM Pi, the *phoCΩ490* mutant grew poorly, however the double mutants carrying *phoCΩ490* together with *phoU10, phoB8, phoB3* or the *sfx2* alleles grew as well as the wild type strain Rm1021 (Fig. 5-6). This result is consistent with the finding that strains carrying *phoB, phoU* and *sfx2* mutations in an otherwise wild type background
Fig. 5-6 Growth experiment showing suppression of the growth phenotype of phoC mutant in MOPS-buffered minimal media containing 2mM Pi by *R. meliloti* phoUB mutants. The strains presented are Rm1021 (wt, ■), RmG490 (phoCΔ490, ○), RmH625 (phoB3, phoCΔ490, ◊) and RmH838 (phoB3, ◆). The growth characteristics of RmH623 (phoU10, phoCΔ490), RmH624 (phoB8, phoCΔ490) and RmH363 (sfx2, phoCΔ490) were similar to those of RmH625 and are not presented here to simplify the figure. Each data point represents the average of triplicate values.
grew like the wild type strain in media containing 2mM Pi (Fig. 5-4), despite the fact that the *phoCDET* encoded phosphate transport system is not expressed in these strains (Fig. 5-5b). The system allowing Pi transport into *phoUB* mutants could also be responsible for allowing the *phoUB-phoCΩ490* double mutants to grow normally in media containing 2mM phosphate (Fig. 5-6).

Oresnik et al., (1994) observed that *phoCDET* (*ndvF*) mutants form mucoid colonies when plated on low osmolality GYM medium whereas the wild type formed non-mucoid (dry) colonies on this medium. The mucoid phenotype is dependent upon genes required for synthesis of the exopolysaccharide II (EpsII) of *R. meliloti* and these genes were known to be expressed under phosphate starvation conditions (Zhan et al., 1991). This mucoid colony phenotype was also reversed by increasing the osmolarity of the media by adding 100mM of various salt, such as NaCl, KCl or MgSO₄. In addition *phoCDET sfx1* or *phoCDET sfx2* double mutants generated a dry wild type phenotype (Oresnik et al., 1994). We investigated whether the *phoUB* mutations were able to suppress the mucoid phenotype of *phoCDET* mutants by plating RmH623 (*phoCΩ490 phoU10*), RmH624 (*phoCΩ490 phoB8*) and RmH625 (*phoCΩ490 phoB3*) on GYM agar. In all cases a dry colony morphology comparable to that of the wild type was obtained (Table 5-4). Moreover when the *phoCΩ490* mutant RmG490 was plated on GYM agar supplemented with 2mM AEP (a Pi source the mutant strain was able to utilize), the colonies had a dry wild type morphology. These data
<table>
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<td>D</td>
</tr>
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**Table 5-4:** Colony morphologies of sfx2, phoU10, phoB8 and phoB3 mutants in wild type and phoCφ490 backgrounds on low osmolarity plates (GYM; glutamate / yeast / mannitol) and GYM plates supplemented with 100mM NaCl. Rm1021 and RmG490 were also tested on GYM media containing 2mM aminoethylphosphonate (AEP2). D and M indicate a dry-type and a mucoid-type colony morphology, respectively.
provide evidence that the mucoid phenotype of \textit{phoCDET} mutants is a direct consequence of the phosphate starvation state of the cells.

In summary, mutations at the \textit{phoUB} locus were phenotypically similar to the \textit{sfx2} suppressor mutation and suppressed all of the known phenotypes associated with mutations in the \textit{phoCDET} locus.

8- \textit{phoUB} and \textit{sfx2} mutations lead to increase in \textit{orfA pit} expression

In view of above results, we suspected \textit{phoB} and/or \textit{phoU} mutations to increase expression of the recently characterized \textit{orfA-pit} locus which appears to encode an alternate phosphate transport system in \textit{R. meliloti} (see Chapter IV). To investigate this possibility, we transduced \textit{phoB}, \textit{phoU} and \textit{sfx2} mutations into the chromosomal-borne transcriptional \textit{lacZ} fusion, to the wild type-\textit{pit} (strain RmH754 (\textit{phoU10}), RmH755 (\textit{phoB8}), RmH756 (\textit{phoB3}) and RmH765 (\textit{sfx2})) and to the \textit{sfx1-pit} locus (strain RmH861 (\textit{phoU10}), RmH862 (\textit{phoB8}), RmH863 (\textit{phoB3}) and RmH860 (\textit{sfx2})). The level of \textit{pit} expression (\(\beta\)-galactosidase activity) was measured after 32 hours growth in MOPS P0 and MOPS P2. The results from these experiments are shown in Figure 5-7a and b. As previously reported (Chapter IV): i) the overall levels of \textit{pit} expression were higher when directed from the \textit{sfx1} promoter than when directed from the wild type \textit{pit} promoter; ii) in the wild type background, \textit{pit} expression directed from both wild type and \textit{sfx1} promoters was phosphate regulated as shown by the \(\beta\)-
Fig. 5-7 Alkaline phosphatase (a) and β-galactosidase (b) activities of wild type- and sfx1-pit::lacZ chromosomal fusion in Lac−, wild type (wt), phoU10 (U10), phoB8 (B8), phoB3 (B3) and sfx2 backgrounds. The assay was performed after 32 hours growth in MOPS-buffered minimal media with no phosphate added (MOPS P0, solid box) or in media supplemented with 2mM Pi (MOPS P2, open box). Each value represents the average of triplicate values ± S.E.
galactosidase activity which was 3- to 4-fold higher in cells grown with 2mM phosphate compared to phosphate limited cells (P0). The results also demonstrate: iii) in the phoU/phoB/sfx2 backgrounds, pit expression was increased 4.5- to 5.5-fold when the cells were grown in MOPS P0 and 1.5- to 2-fold when the cells were grown in MOPS P2 compared to its expression in a wild type background suggesting that phoB (phoU) is negatively regulating pit expression; iv) in the phoU/phoB/sfx2 backgrounds the phosphate dependent regulation of pit expression from a wild type promoter was dramatically reduced with the expression of pit in MOPS P0 nearly reaching the expression in MOPS P2 grown cells, suggesting that phosphate regulation of pit expression was probably mediated via PhoB (PhoU). The same tendency was also observed in a sfx1-pit::lacZ fusion but not as clearly.

A similar regulation was observed when the plasmid fusions were mated into (1) phoUB(sfx2) backgrounds (data not shown) and (2) phoCΩ490/phoUB (sfx2) backgrounds (Fig. 5-7c and d) suggesting that the increase of pit expression in phoUB/sfx2 mutant strains was independent of phoCDET. Suppression of phoCDET mutations by phoUB and sfx2 then appear to be due to a 7- to 10-fold increase in pit expression (determined by comparing the difference in pit expression between phoC and phoC phoUB/sfx2 mutants from MOPS P2 grown cells). The higher level of pit repression in cells grown under phosphate limiting conditions is believed to be due to the larger amount of
Fig. 5-7 Alkaline phosphatase (c) and β-galactosidase (d) activities of pit::lacZ plasmid fusion in Lac" wild type (wt), phoCΩ490 (phoC), phoU10/phoCΩ490, phoB8/phoCΩ490, phoB3/phoCΩ490 and sfx2/phoCΩ490 backgrounds. The assay was performed after 38 hours growth in MOPS-buffered minimal media with no phosphate added (MOPS P0, solid box) or in media supplemented with 2mM Pi (MOPS P2, open box). Each value represents the average of triplicate values ± S.E.
activated PhoB protein present in these cells. A non-negligible level of activated PhoB (PhoU) protein appeared to be present in MOPS P2 grown cells as PhoB was able to repress expression of the pit locus to up to two fold.

Expression of the wild type and sfx1-pit alleles showed homologous response to the phoUB-sfx2 mutations suggesting that the sfx1 mutation did not affect the possible PhoB binding site in the promoter region of the orfA-pit locus. Although, sfx1 on one hand and sfx2/phoUB on the other hand suppressed phenotypes associated with mutation in the phoCDET locus by increasing pit expression the mechanism by which this occurred appeared to be distinct.

9- orfA-pit::Tn5 phoB/sfx2 double mutations are lethal

The absence of a functional PhoCDET transport system in a phoUB mutant suggested that phosphate was assimilated via an alternative transport system such as Pit. The genetic evidence above is consistent with this hypothesis. Here, we present further evidence which stems from our finding that we were unable to construct a phoB3 orfA/pit::Tn5 or sfx2 orfA/pit::Tn5 double mutants (Table 5-5). Strains RmG765 (orfAΩ2.3::Tn5) and RmG774 (pitΩ23.10::Tn5) were used as donors to transduce the orfA pit alleles into RmH406 (phoB3) and RmG497 (sfx2) strains. Transductants were selected on LB medium containing 200 μg/ml neomycin. Both orfA/pit::Tn5 alleles were
Table 5-5  Viability of sfx2 and phoB3 strains after transducing lysates made from two sfx1::Tn5 insertions strains in the absence or the presence of pTH90 (sfx1) or pTH38 (phoCDDET). Viable counts of the transduction mixtures were determined to ascertain that the number of recipient cells were equivalent for every transduction. The number (#) of Nm transductant was determined after plating 0.1ml of the transduction mixture on selective plates. ND: not determined.
<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Viable counts (x10^6 cells)</th>
<th># of Nm&lt;sup&gt;r&lt;/sup&gt; transductants (per 0.1ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RmG765</td>
<td>Rm1021 wt</td>
<td>131</td>
<td>167.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>RmH852 phoB3::Tn5-233</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmH856 phoB3::Tn5-233 / pTH90 (sfx1)</td>
<td>138</td>
<td>74.0 ± 3</td>
</tr>
<tr>
<td></td>
<td>RmH855 phoB3::Tn5-233 / pTH38 (phoCDET)</td>
<td>113</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmG497 sfx2, Ω5033::Tn5-233</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmH503 sfx2, Ω5033::Tn5-233</td>
<td>69</td>
<td>26.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>RmH502 sfx2, Ω5033::Tn5-233 / pTH38 (phoCDET)</td>
<td>124</td>
<td>0</td>
</tr>
<tr>
<td>RmG774</td>
<td>Rm1021 wt</td>
<td>64</td>
<td>210.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>RmH852 phoB3::Tn5-233</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmH856 phoB3::Tn5-233 / pTH90 (sfx1)</td>
<td>156</td>
<td>90.0 ± 4</td>
</tr>
<tr>
<td></td>
<td>RmH855 phoB3::Tn5-233 / pTH38 (phoCDET)</td>
<td>194</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmG497 sfx2, Ω5033::Tn5-233</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RmH503 sfx2, Ω5033::Tn5-233 / pTH90 (sfx1)</td>
<td>51</td>
<td>40.0 ± 3</td>
</tr>
<tr>
<td></td>
<td>RmH502 sfx2, Ω5033::Tn5-233 / pTH38 (phoCDET)</td>
<td>136</td>
<td>0</td>
</tr>
</tbody>
</table>
readily transduced into the wild type strain Rm1021, however no transductants were obtained with the phoB or sfx2 recipient strains unless these strains were made meridiploid for the orfA pit locus by first introducing the pTH90 plasmid. Making the phoB or sfx2 mutants meridiploid for the phoCDET locus by transferring pTH38 had no effect, presumably because phoCDET expression occurs neither in a phoB or sfx2 background. The requirement of a functional Pit protein for the survival of phoB and sfx2 mutants suggests that the alternate Pi transport system which must be operative in these mutants is Pit protein dependent.
C. Discussion

Genetic analysis of the sfx2 suppressor mutation resulted in the isolation of one phoU and two phoB transposon insertion mutants of R. meliloti. The precise location of the insertions within the phoB and phoU structural genes were established by DNA sequencing. The three mutants were identified on the basis of their alkaline phosphatase negative (AP−) phenotypes and their linkage to the sfx2 locus. In the case of the phoB mutants, the AP− phenotype was expected as PhoB is required for AP expression in other bacteria (Lee et al., 1989; Anba et al., 1990). On the other hand, the AP− phenotype of the phoU mutant was unusual as phoU mutants of both E. coli and P. aeruginosa were constitutive for alkaline phosphatase activity (Steed et Wanner, 1993; Kato et al., 1994, respectively). In R. meliloti the phoB gene is located downstream from phoU (accession # M96261 Cannon et al., unpublished). Given the phoUB gene order and the relatively small (111 bp) intergenic region, it is possible that in R. meliloti, phoUB are transcribed as a single mRNA, in which case phoU insertion mutants would also be genotypically phoB−. If this is the case, elucidation of the role of phoU in R. meliloti will require the construction of defined phoU phoB+ strains. It is worth noting the E. coli phoU mutants grow poorly in MOPS P2 and M63 media containing a variety of carbon sources as well as in complex media (Steed
and Wanner, 1993). We haven't observed analogous phenotypes with the *phoU* insertion mutant we isolated in *R. meliloti*.

Analysis of the sequence downstream of *phoB* revealed no *phoR*-like open reading frame, suggesting that *phoR*, if present in this strain, does not form an operon with *phoB* as observed in various organisms (Lee et al., 1989; Anba et al., 1990 and Lee and Hulett, 1992). We also note that our screening experiment did not result in the isolation of any *phoR* mutants. It is, however, possible that one would not detect *phoR* mutants as AP\(^{+}\) colonies if PhoB can be activated by kinases other than PhoR; thus leading to PhoR-independent transcriptional activation of genes of the Pho regulon. Kinases other than PhoR are known to activate PhoB in *E. coli* (Amemura et al., 1990; Wanner, 1996).

Our data showing that *phoD* and *phoE::lacZ* gene fusions are not expressed in a *phoB* mutant background (Fig. 5-5) is consistent with the presumed role of the *R. meliloti* PhoB protein as the central transcriptional activator of genes whose expression is responsive to low levels of extracellular phosphate. We previously identified two *E. coli* "Pho Box"-like sequences overlapping the -35 region of the *phoC* promoter (Bardin et al., 1996). By analogy to what is known in *E. coli*, it is likely that the *R. meliloti* PhoB protein binds the "Pho Box" elements at the *phoC* promoter to activate its transcription.

The observation that *sfx2* and *phoUB* insertion mutants behaved like a wild type strain when inoculated in plants or when grown in 2mM Pi, despite the
lack of \textit{phoCDET} expression, suggested that an alternative Pi transport system was induced in these mutants. In addition, the loss of viability of \textit{phoUB/sfx2 orfA/pit::Tn5} double mutants prompted us to monitor \textit{pit} expression in a \textit{phoUB/sfx2} mutated background. In a wild type background, \textit{pit} expression, from chromosomal \textit{lacZ} fusions, was higher (although weakly; 2-fold) under conditions of excess Pi than under conditions of phosphate starvation (Fig. 5-7, see also Chapter IV). In the \textit{phoB} background, however, \textit{pit} expression showed little to no regulation by the phosphate concentration of the media (similar level of \textit{pit} expression whether the cells were grown in MOPS P0 or MOPS P2), and the overall level of \textit{pit} expression was higher in this background than in a wild type background. Thus, in contrast to \textit{phoCDET} transcription, our data employing \textit{pit::lacZ} gene fusions suggest that PhoB, or a PhoB-dependent gene product is a negative regulator of \textit{pit} expression. PhoB-dependent regulation of \textit{pit} expression therefore contrasts with the constitutive expression of the Pit transport system of \textit{E. coli} (Rosenberg, 1987). It is interesting that expression of \textit{pit} and \textit{phoCDET} encoded Pi transport systems show opposite patterns of expression in response to concentration of available phosphate. It is likely that the pattern of \textit{phoCDET} and \textit{pit} expression reflects different physiological characteristics of the two transport systems. Indeed, we have recently obtained evidence that the Pit system is a low-affinity Pi transporter, whereas, the
PhoCDET system has a high-affinity for Pi (Voegele, Bardin and Finan, 1997 in preparation).

Most studies of the Pho regulon have focused on genes whose expression increases in response to Pi limitation. VanBogelen et al. (1996) recently published a survey of *E. coli* proteins whose synthesis was influenced by the extracellular phosphate concentrations. These authors estimated that the synthesis rate of 413 proteins was modified under phosphate limitation, of these 208 were induced and 205 repressed. These authors noted that the promoter regions of three repressed genes, *ompF*, *pfl* and *ssb* contain putative PhoB boxes, however other than that observation we are not aware of any reports where the actual mechanism of repression of these genes has been examined. Smith and Payne (1992) suggested that PhoB may repress expression of periplasmic peptide transport binding proteins under low phosphate conditions. They also identified a putative PhoB Box that seemed to overlap with the RNA polymerase binding sites of normal promoters. The work presented here then provides the first genetic evidence of a PhoB or a PhoB-dependent protein acting as phosphate-dependent repressor of gene expression. The mechanism by which available Pi regulates *orfA pit* expression will require further analysis of the promoter region; in this respect it is interesting that the *sfx1* mutation increases the basal rate of *pit* transcription, yet *pit* expression from the *sfx1* locus is still regulated by the concentration of available phosphate. PhoB inactivation
abolishes phosphate regulation of the wild type- but not of sfx1-orfA pit locus. The sfx1 mutation then “creates” a new phosphate regulation of the locus which is not PhoB-dependent. The increase in pit expression by sfx1 and sfx2/phoUB mutations therefore occur via two different mechanisms.

It is worth mentioning that a significant amount of activated PhoB protein seems to be present in cells grown under phosphate sufficient conditions as pit expression under these growth conditions was repressed up to two fold compared to its expression in a phoB' background. As a pit' mutant showed no growth or symbiotic phenotypes (data not shown), we argue that the level of phoCDET expression in MOPS P2 may be sufficient to assume normal functioning of the cells.

PhoB was required for growth of R. meliloti in MOPS-buffered minimal media containing various phosphonates as the sole source of P (Fig. 5-4). In E. coli the uptake and/or degradation of aminomethylphosphonate, methylphosphonate and ethylphosphonate by the C-P lyase pathway is under Pho regulon control (Wackett et al., 1987). Thus the requirement of a functional PhoB protein for growth on these compounds was expected with R. meliloti as well. It was however surprising that the phoUB mutants grew as well as the wild type (after a slight delay) when aminoethylphosphonate (AEP) was the sole P source (Fig. 5-4). This compound can be taken up and metabolized by the C-P lyase pathway in E. coli and the phosphonatase pathway in Enterobacter
*aerogenes* and *S. typhimurium*. In these last two organisms, expression of the phosphonatase pathway was also activated by PhoB (Lee et al. 1992; Jiang et al. 1995). Our study clearly demonstrates that *R. meliloti* can transport and metabolize AEP via a PhoB-independent pathway.

*sfx1* and *sfx2* were originally identified as mutations which suppressed the symbiotic Fix* phenotype of *ndvF (phoCDET)* mutants. The *sfx1* mutation was previously shown to result in increased expression of the *orfA pit* operon. As *pit* is homologous to many phosphate transport proteins (see Chapter IV), we hypothesize that the increased expression of *pit* results in increased phosphate transport and hence suppression of the growth and symbiotic phenotypes of the *ndvF (phoCDET)* mutants. Our findings that *sfx2* and *phoU/B* mutations result in increased *pit* expression are also consistent with the idea that increased expression of *pit* is responsible for the suppression of the symbiotic and free-living phenotypes associated with *phoCDET (ndvF)* mutations. The dominance of the *sfx2* mutation may be attributed to the fact that this mutation leads to the formation of a modified protein that impairs with the functioning of the wild type protein. The modified protein may have increased affinity for the promoters of genes of the Pho regulon, preventing access of the wild type protein and thus transcriptional activation of the genes. Another possible explanation may lay in the structure of the protein; if the protein is active as a dimer, the formation of an
hetero-oligomeric structure made of wild type and modified peptides could form an inactive protein, here again preventing expression of the Pho genes.
Appendix C

- **Appendix C-1:** Alkaline phosphatase activity of sfx2 and sfx3 strains.

  The strains tested were 1021 (wt), RmG439 (ndvFΔG439), RmG514 (ndvFΔG439, sfx2, Ω5025::Tn5), RmF114 (ΔΩ5033-5064::Tn5-233; megaplasmid deletion removing the phoCDET locus (Fig. 1-2, Top)), RmF346 (ΔΩ5033-5064::Tn5-233, sfx2; strain isolated from Fix⁺ nodules) and RmG425 (ΔΩ5033-5007::Tn5-233, sfx3, strain isolated from Fix⁺ nodules).

⇒ No AP activity in sfx2 and sfx3 containing strains.
• Appendix C-2: Map of The *phoUB* locus of *Rhizobium meliloti* showing the TnV subclone of *phoU10* (pTH292), *phoB3* (pTH287) and *phoB8* (pTH311). The four sequenced fragments are indicated:

Sequence 1: determined from pTH301 (pTH292 subclone (*phoU10*))

Sequence 2: determined from pTH328 (pTH292 subclone(*phoU10*))

Sequence 3: determined from pTH302 (pTH287 subclone (*phoB3*))

Sequence 4: determined from pTH331 (pTH311 subclone (*phoB8*))

Restriction sites indicated: *HindIII* (H); *SalI* (SI).
• **Appendix C-3**: Phenotypes associated with the RmF222 mutant.

RmF222 is a Rm1021 strain mutated with ethyl methanesulfonate which showed reduced AP activity (Long et al., 1988). In LB, the mutant strain grew as well as the wild type parent strain (Rm1021) and was able to effectively nodulate alfalfa plants. As it is believed that this mutant probably affects a regulatory gene of the Pho regulon, we investigated whether it was linked to the *phoUB* locus.

• Linkage experiments:

We transduced the Nm' of Ω5258::Tn5 (blue on X-Phos) into RmF222 and obtained a 56.5% linkage (26 blue colonies out of 46 colonies patched); a linkage similar to the one obtained when Ω5258::Tn5 was transduced into *phoUB/sfx2* mutants. In addition, pTH282 and pTH284, plasmids complementing the AP phenotype of *phoUB/sfx3* mutants, were also able to complement the AP phenotype of RmF222.

• In contrast with *phoUB/sfx2* mutants, F222 mutation did not suppress the *phoCDE* symbiotic phenotype and exhibit a different growth pattern than *phoUB/sfx2* mutants when grown in media containing various phosphorus:

The growth over time of RmF222 (◇) in, MOPS P0, and 2mM of Pi, AEP, EP, AMP, MP, G3P, Glc6P and PSer, is compared to the wild type strain Rm1021 (■) and the *phoCΩ490, RmG490* strain (●).
* In MOPS P0, after delay, F222 reached the wild type growth background. In the same media, \textit{phoUB/sfx2} mutants showed no growth.

* In MOPS Pi, F222 showed intermediate growth between the wild type strain and the \textit{phoC} mutant. In the same media, \textit{phoUB/sfx2} mutants grew as well as the wild type strain.

* In MOPS AEP, F222 showed delayed growth compared to the wild type and \textit{phoC} mutant as observed for the \textit{phoUB/sfx2} mutants.

* In MOPS EP, F222 started growing 50 hours after the wild type strain; very little growth was observed in this media for the \textit{phoUB/sfx2} mutants.

* In MOPS AMP, F222 reached the wild type growth after 30 hours delay; very little growth was observed in this media for the \textit{phoUB/sfx2} mutants.

* In MOPS MP, F222 started growing 75 hours after the wild type strain; no growth was observed in this media for the \textit{phoUB/sfx2} mutants.

* In MOPS G3P, F222 like the \textit{phoUB/sfx2} mutants showed growth delay (about 5 hours) compared to the wild type strain.

* In Glc6P, F222 showed a slight growth delay compared to the wild type strain as observed for the \textit{phoUB/sfx2} mutants.

* In MOPS PSer, the growth of F222 strain was slower that the \textit{phoUB/sfx2} mutants.
• **Appendix C-4:** Sequences of *pho27* from both side of the TnV insertion.

Note: J111 and J112 correspond to the E. coli strain numbers containing the two subclones.

>**J111**

CCATCGATGCGAAGGCCGTCTCTGGTCTCGACCAGCGGCAACGCAGAAAGGACACGG
GACGAACCCGACGGGTCTCCGGCGCGAGACCGTGCGCAGCTCCGACCCCGACCCCGACCCTCGAAAGC
TGTTCTTCCGCACCGGCGGCGGCGCGACGAGATGCGCGAACCACCAGTCGAGCGGACG
ACACCTTCTCCCTCGGCTGCTACATCGGCGGATCGGCGTGCTCGAACCACCTACGAAAG
CGCGCAAACGGTGGCCCGAGATTTCCCGCGACgACATGGCCGTTGCGGCCCGcCTGTGCCTCGG
TGACCAgGCAGGGTGGCGCGCAAGATGCGCTGAAAACCAGGCTGAAgAGTTCCGGAGCGCGC
CTGGCGJAGCCGcCTCAGGCGCAAGGCTCTGCTGATGCCTCGGGTgAGCTGGCCCTTCTCTCGC
TCGGTC3TTCCCTGTGCTGTCACagGGATCCAGGAgACCagGTCTCTnGAGCTGAAaAGA
TTCTCCCGCGCGAGCGCGAGGAAGACTGCGTTAGCTGAGTTCCGGTGGTNNACTGGGGGNAGANTACGCTCNAAG
CGAGGCCGTAAGNTCTTNCCCTCTCCTCCTCNCNCCAGCTTCCGAANNCAACTTTAGGTTGNCN
CTTTCCTTTNCNCCNTTCC

>**J112**

CATCGATGGCGCAATTTGTCCGGCGATGCCGAGACCGTGCTCGGCTCGGCGGTGAGA
AgGAgGCACCCGACCTCAGGGCGAGCTCGGAGATCCGGACCCACCTACGACGATCGCCTCCAGCGG
ATTTCGACTGAGGGCCAGATGCGCCGCTCCGTTTCCGGAAATCTCAGGACGATGCGGCGGAGACAT
CTGGCGACCCGATTGCCGCGCTCGCGCTTCTCGCTCCTTGGCTGTTGGTCGAC
CATGACATAGACCTTGCAGGCGTCTCGGGTGGTGGCTGGAGCTCTCCGGCGGCGGCTCCACATCTTT
GGTCGGCCCGACGCCATCGGGCGCAAGAGGGGTGTCCTACAGCAGCCTCGCGGCGCTGGAGGCC
GAAGGGTGTCTCGGGCGTCAGGGACCTTCCGGCAGATGGAGAGGGAGATCCGAGTGGACGt
GGCGTCTCCTCGGAGACTTGGCGCAGTGAGGCTCTCATCGGAAgAGATACTATATCgC
GCGCGGTCTCgCcaGGTTGTAATGCTCCCTGTCGTCAGAACCGTTGATTGACTAGTcCgTAAC
CTGTGGTGCgCGCTATaTAGAAGANCCNCGGTCCCTGTGAGNATCNACTTCCACCT
CGGTCTCTGAAACGTCTNCCCTCGGCGCTCCTCNGAAGTCCTTTCCCTGNAAAT
TCNACANTCTNCTGCCCCCGGNTCNCNAGGCCTTTCCCTGNAAATTCTCACTNCTCTACT
NTTNCTTNNTNTNCTNGCCCNANTCCNCNCTCNCNCTCAACGGNAACTCNCTCC
Chapter VI

General Discussion

A) The new symbiotic locus \textit{ndvF} encodes a phosphate transport system in \textit{Rhizobium meliloti}.

The \textit{ndvF} locus consists of four genes, \textit{phoCDET} which encode an ABC-type transporter homologous to the \textit{phnCDE} phosphonate transport system of \textit{Escherichia coli} (Metcalf and Wanner, 1991). The PhoC protein is homologous to PhnC, the ATPase component of the transporter. PhoD is similar to PhnD, the putative periplasmic substrate-binding protein. The hydrophobic nature of PhoE and PhoT, both homologous to PhnE, make them likely to constitute the transmembrane components of the transporter. Evidence suggesting that \textit{phoCDET} encodes a phosphate transporter include:

- \textit{phoCDET} mutants grow poorly in MOPS-buffered minimal media containing 2mM Pi.
- \textit{phoCDET} mutants fail to transport phosphate at low concentration.

In addition, all the phenotypes associated with the \textit{phoCDET} mutations were shown to be the result of phosphate deprivation in those strains:
1. The high level of alkaline phosphatase (AP) activity measured in *phoCDET* mutants cells, even when the cells were cultured under phosphate sufficient conditions (conditions that repress AP expression in wild type cells), was attributed to the phosphate-starvation state of these cells. AP expression was indeed repressed when these mutants strains were grown in media supplemented with phosphorus sources they can utilize (such as AEP (Fig. 5-1), G3P, Glc6P and PSer; data not shown).

2. The mucoid phenotype of *phoCDET* mutants when plated on the low osmolarity media (GYM) is dependent on the expression of the *exp* genes encoding for the synthesis of exopolysaccharide II (Oresnik et al., 1994). Induction of these genes under phosphate limitation (Zhan et al., 1989; 1991) as well as reversion of the mucoid phenotype of *phoCDET* mutants to a dry (wild type) phenotype by adding 2mM AEP to the GYM plate also pointed to the phosphate starved behavior of these cells. It is interesting to mention that increasing the osmolarity of the GYM media by adding 100mM of various salts also reverted *phoCDET* mutants to a dry phenotype. This effect is likely due to the repression of the *esp* genes synthesis rather than a direct effect of the salt concentration on the phosphate transport system; the 100mM NaCl present in the MOPS P2 media did not prevent the growth phenotype of these mutants.
3. The symbiotic phenotype of phoCDET mutants was suppressed by spontaneous second site mutations in two distinct chromosomal loci both of which increased expression of an alternative phosphate transport system homologous to pit, the low-affinity phosphate transport system in E. coli (Sofia et al., 1994). The two types of suppressor mutations named sfx1 and sfx2, also suppressed all the phenotypes associated with the phoCDET mutations. This provides definite evidence that the Fix+ phenotype of phoCDET mutants was the result of the inability of these mutants to assimilate phosphate.

Kinetic experiments suggest that PhoCDET is a high-affinity, low-velocity transport system. The nature of the phosphate uptake inhibition in presence of phosphonates and arsenate has not been characterized yet. A competitive inhibition would suggest that these compounds are transported into the cell by the PhoCDET system. This would imply that PhoCDET has a wide substrate-specificity which contrasts with the high-specificity for phosphate of Pst, the high-affinity phosphate transporter of E. coli. Bacteria may have evolved such a transport system to cope with the soil environment where compounds like phosphonates may provide an alternative source of phosphorus.

Many bacteria have high-affinity phosphate transport systems and genetic characterization of some of these transporters indicates that they are ABC-type homologous to the pst system of E. coli (Nikata et al., 1996, Takemaru et al.,
1996). The ABC-type transporter appears to provide the best mechanism to assimilate a solute against a very high gradient.

B ) An alternative phosphate transport system in R. meliloti.

Subcloning and sequencing of the sfx1 suppressor locus revealed the presence of two open reading frames which partially overlap and thus likely form an operon. The deduced protein of the first gene, orfA, showed no significant homology with proteins deposited in data banks while the second gene was homologous to pit, the low-affinity phosphate transport system of E. coli as well as other phosphate transporters found in both prokaryotic and eukaryotic organisms (see results section of Chapter IV). Suppression of the phoCDET associated phenotypes by the sfx1 mutation resulted from a thymidine deletion in a seven T stretch lying 80 nucleotides upstream of the presumed start codon of orfA. This mutation seems to be responsible for increased expression of the orfA-pit genes. Increased expression of orfA pit, either via the sfx1 mutation or by increasing the copy number of the wild type allele (by subcloning the locus on a multicopy plasmid), was sufficient to allow enough phosphate uptake (Dr. Voegele, personal communication) to restore normal growth in MOPS P2 and
the symbiotic capability to the *phoCDET* mutant strains. The role and function of *orfA* remains unknown.

The presence of two phosphate transporters in *R. meliloti* 1021 conflicts with the results previously reported which indicated that various *Rhizobium* strains carried only a single phosphate transport system (Smart et al., 1984a). *E. coli* (Willsky and Malamy, 1980a), *Acinetobacter johnsonii* (Van Veen et al., 1993a), *Acinetobacter lwooffi* (Yashphe et al., 1992) and *Bacillus cereus* (Rosenberg et al, 1969), however, possess two main Pi transport systems that differ in their affinity and specificity for phosphate as well as for the type of phosphate species they transport (as observed in *E. coli* and *A. johnsonii*, Van Veen et al., 1993b and 1994b). This suggests that these organisms have evolved two Pi transport systems in order to adjust to the phosphate species found in their environment. While the PhoCDET transport system appears to be essential for the growth of *R. meliloti* in the low-phosphate soil environment and nodule induction, the use of a Pit-like system in this bacteria remains to be defined.
C) **Regulation of the phosphate transport systems.**

Both *phoCDET* and *pit* expression appeared to be phosphate regulated via the PhoB (and possibly PhoU) protein but in opposite manners. Expression of *phoCDET* is induced and *pit* expression is repressed under phosphate-starvation conditions by PhoB or a PhoB-dependent protein. Regulation of the phosphate transport systems in *R. meliloti* 1021 is proposed in the following model (Fig. 6-1): When the cells are grown under phosphate deprivation, the PhoB protein, probably activated by phosphorylation, induces expression of the Pho regulon, including *phoCDET*, and represses either directly or indirectly expression of the *orfA-pit* locus. High phosphate conditions on the other hand, either prevent PhoB expression or deactivates PhoB leading to derepression of the *orfA-pit* locus with a concomitant decrease in *phoCDET* expression. This model is supported by the recent finding that Pit appears to be the main phosphate transport system in wild type cells grown in MOPS media containing 2mM Pi (Dr. Voegele, personal communication).

In accordance with the proposed model, the slow growth phenotype of *phoCDET* mutants in MOPS-buffered minimal media containing 2mM Pi can be interpreted by the fact that in these mutants the high content of activated PhoB, as suggested by the high AP activity, results in *pit* repression (as seen by the decrease in *pit* expression in a *phoCDET* mutant background; Fig. 5-7d). The
Fig. 6.1: Model showing the regulation of phoCDET and orfA pit loci under low and high phosphate conditions. Under low phosphate conditions, we believe that expression of the phoUB operon is induced. The PhoB protein produced is probably activated by phosphorylation (as shown in E. coli) and the activated protein (PhoB\textsuperscript{A}) induces phoCDET expression. PhoB, either directly or indirectly (via a protein X), also represses orfA pit expression. Under high phosphate conditions, PhoB\textsuperscript{A} is not produced due to either to:

1) repression of the phoUB operon by the high Pi environment; or
2) the activated PhoB protein is not produced.

In any case, this results in the absence of phoCDET expression and in the derepression of orfA pit leading to the expression of these genes.
Low Pi conditions

phoUB operon

PhoB

PhoB*

phoCDET

orfA pit

High Pi conditions

phoUB operon

PhoB

phoCDET

orfA pit
PhoCDET system then appears to be required for Pi signaling. A similar role has been proposed for the PstSCAB Pi transport system of *E. coli* (Wanner, 1996). The slow growth of these mutants is then indicative of slow phosphate uptake which is partially due to residual activity of Pit but also to alternative low-affinity phosphate transporter(s) as the double mutant (*phoCDET, pit::Tn5*) still grew but at a slower rate than a *phoCDET* mutant (Fig. 4-4b). In *E. coli*, alternative phosphate uptake systems include the phosphonate, the glycerol-3-phosphate and the glucose-6-phosphate transport systems (Metcalf and Wanner, 1991; Maloney et al., 1990). Systems similar to some of these phosphorus transporters are likely to be present in *R. meliloti*. These alternative phosphate transport systems appear to be PhoB regulated because the *phoB/pit* double mutation (in which *phoCDET* is not expressed) was lethal while *phoCDET/pit* double mutants were not.

Regulation of the phosphate transport systems in *R. meliloti* contrasts with the ones in *E. coli*, *A. johnsonii* and *P. aeruginosa* (Willsky and Malamy, 1980a; Van Veen et al., 1993a; Poole and Hancock, 1984) as far as the expression of the low-affinity phosphate transport system is concerned. We demonstrated that the *pit* system of *R. meliloti* is phosphate regulated by a PhoB-dependent mechanism while the low-affinity transporters are believed to be constitutively expressed in these organisms.
D) **Importance of the phosphate transport systems for the establishment of an efficient symbiosis.**

Because of the competition between microorganisms for nutritional resources in the soil, bacteria equipped with an efficient uptake system for an essential compound which is present in low concentrations in soil, such as phosphate, will certainly have an advantage over strains with a less efficient uptake system. Growth efficiency is however not the only factor influencing the establishment of an efficient symbiosis. An increasing number of articles suggests a probable effect of P deficiency on the release of Nod factors and modification of molecules (lipopolysacharides) that are at the base of the communication between the plant host and rhizobia for the establishment of an efficient symbiosis (Mckay and Djordjevic, 1993; Howieson et al., 1993; Tao et al., 1992). Strains deficient in phosphate uptake may then have the dual disadvantage of growing poorly in the soil environment and be impaired in the release of Nod factors or in the synthesis of molecules essential for initiation of the infection process. In the case of the *phoCDET* mutants, reduction in phosphate uptake primarily affects the infection process as numerous small white nodules were present on the root of alfalfa plants inoculated with these mutant strains, although after delay. Due to the importance of a high affinity phosphate transport system for viability in the soil environment and the
establishment of an efficient symbiotic interaction with the plant host, it is not surprising that bacteria carrying second-site mutations which activate an alternative Pi transport system are readily selected for by the plant host.

Although, we are not able to investigate whether the high-affinity phosphate transport system is required in the nodule environment, it is rather unlikely due to the very high phosphate content as confirmed by, the presence of polyphosphate granules in the nodules and the low level of phosphate uptake measured in snake bean bacteroids (Smart et al., 1984a). Mutations in the pit locus show no effect on either the efficiency of nodule infection or acetylene reduction activity (data not shown). This suggests that pit is not required in the nodule or that the low level of phoCDET expression in this high phosphate environment was sufficient to take over pit deficiency. Phosphate uptake by phoCDET was indeed increased when cells lacking the Pit system were grown in MOPS 2mM Pi (Dr. Voegele, personal communication). It is interesting that Smart et al. (1984a) noted that AP activity in the bacteroid was significantly higher than in free-living bacteria grown under phosphate-rich conditions. This activity may however be due to factors independent of the phosphate concentration in, or surrounding the bacteroids.
References


