REGULATION OF *PSTSCAB-PHOUB* GENES IN *SINORHIZOBIUM MELILOTI*

Regulation of *pstSCAB-phoUB* Genes

In *Sinorhizobium meliloti*

By

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ABSTRACT

Previous studies in this laboratory have identified two phosphate transport systems in *Sinorhizobium meliloti* encoded by the *phoCDET* and *orfA-pit* genes respectively. The PhoB regulatory protein is required for transcriptional activation of the *phoCDET* genes but repress the transcription of *orfA-pit*. Determination of the DNA sequence upstream of *phoU-phoB* revealed the presence of genes homologous to the *pstA-pstB* genes, which encode components of an ABC-type high affinity Pi specific transport system in *E. coli*. Further analysis of sequence from the *S. meliloti* genome project (unpublished) revealed the *phoR-pstS-pstC* genes upstream of *pstA-pstB*. Using an R-prime approach, we cloned a 7.5 kb *Hind*III gene fragment which included the above *phoR-pstS-pstC-pstA-pstB* and partial *phoU* genes. Using Tn*5*-B20 and *lacZ-aacc1* cassette gene disruption/fusions, we mutated *pstA, pstB and phoR* gene respectively. We found that: a) *pstA-pstB-phoU-phoB* are in one operon, b) *pstB* expression is not regulated by the media phosphate concentration and is independent of *phoB*, c) in free-living cells, *pstB* mutants, like *phoU* or *phoB* mutants, exhibit alkaline phosphatase negative phenotypes, d) in plant tests, a *pstB* mutant had normal nitrogen fixation ability and like *phoB* mutations, the *pstB* mutation suppressed the Fix^- phenotype of *phoCDET* mutants, e) *phoB* expression is neither regulated by phosphate concentration nor does its expression appear to be auto-regulated, and f) a *phoR* mutant exhibited an alkaline phosphatase negative phenotype. Sequence analysis showed that there is no *pho* box in the upstream of *pstA-pstB-phoU-phoB* operon and the *phoR*, but *pstS* gene has one putative *pho* box in its promoter region. Also discussion and some ideas for future study were presented.

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List of Abbreviations

Ap ampicillin

Bp base pair

Cm chloramphenicol

DNA deoxyribonucleic acid

DNTP deoxynucleotide triphosphate

Gm gentamycin

Kb kilobase or kilobase pair

Km kanamycin

LB Luria-bertani

Nm neomycin

OriV origin of vegetative replication

Ot oxytetracycline

PFU plaque forming units

Rf rifarnpicin

RNA ribonucleic acid

Sm streptomycin

Sp spectinomycin

Tc tetracycline

Wt wild type

X-Gal 5-bromo-4-chloro-3-indolyi-p-D-galactopyranoside

X-Phos. 5-bromo-4-chloro-3-indolyl phosphate.

X-Gluc. 5-bromo-4-chloro-3-indolyl-p-D-glucuronic acid (sodium salt).

^{o}C degree Ceicius

ΩSp spectinomycin resistant interposon

ϕ phage or lysate

^{r} resistant or resistance

ABC type ATP-binding cassette

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CHAPTER I. INTRODUCTION

I-I. Bioligical Nitrogen Fixation.

General Introduction.

Nitrogen is one of the most important elements for organisms. Although gaseous nitrogen (N_2) constitutes 78% of our atmosphere, its inert nature makes it unavailable to eukaryoytic organisms. Bacteria can fix N_2, converting inert N_2 into NH_4^+. Each year, about 175 million metric tons of nitrogen is fixed by bacteria (68%), only 32.3% is produced by industrial processes (Langenheim, 1982). The legume-bacterial symbiosis produces up to 700 kilograms of nitrogen per hectare per year (Evans, 1977). In addition to being cost effective, bacterial nitrogen fixation does not cause environmental pollution.

Depending on the bacteria, nitrogen fixation occurs either in the free-living state or in association with an eukaryotic organism such as in the Rhizobium/legume symbiosis. Some bacteria, for instance, *Azotobacter* can fix nitrogen in free living condition, this process was called nonsymbiotic nitrogen fixation, which can be divided into two groups:

A. aerobic nitrogen fixer.----*Azotobacter*. etc.

B. anaerobic nitrogen fixer----*Clostridium spp*. etc.

Other bacteria, for example, *Sinorhizobium meliloti,* can not fix nitrogen in free living condition, they must associate with host plants to form a symbiosis before they can fix nitrogen. This process was named symbiotic nitrogen fixation. The symbiotic interaction between the microorganism and the eukaryotic host is agronomically important as it enables the host plant to grow in a nitrogen-limited environment, thus

reducing the requirement for nitrogen fertilizer.

Symbiotic Nitrogen Fixation Process.

In symbiotic nitrogen fixation process, bacteria use the fixed nitrogen for their own metabolism, they also supply most of the fixed nitrogen to the host plant. In turn, the host plant supplies the bacterium with C_4 compounds such as succinate, malate and fumarate (Yarosh et al, 1989). Basically biological nitrogen fixation research can be divided into three fields:

A. Bacteria research.

B. Host plant research.

C. Bacteria and plant interaction.

But most of the research conducted in the field of Bacteria. Symbiotic nitrogen fixation in bacteroids is catalyzed by nitrogenase. Bacteria get energy from electrons passed by electron transport chain and break down the $N\equiv N$ N triple bonds to make NH_4^+.

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{nitrogenase}} 2NH_4^+ + H_2 + 16ADP + 16Pi$$

Nitrogenase consists of two components, the homodimeric Fe protein, encoded by *nifH,* and the tetrameric molybdenum (MoFe) containing protein, encoded by *nifD* and *nifK*. The MoFe cofactor is irreversibly denatured by oxygen, which makes nitrogenase highly sensitive to oxygen (Shaw, 1977). On the other hand, there is a high demand for oxygen in nodules as the large amount of energy required by the nitrogenase is generated by oxidative processes and respiration with O_2 as a terminal electron accepter. How do the bacteria solve this problem? It turns out that the leghemoglobin, a kind of protein produced by the host plant, carries the oxygen to the respiration chain and protects the nitrogenase. In the infected cells the oxygen carrier leghemoglobin facilitates oxygen diffusion. 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 (Bergersen F. 1976). The *nif* genes encoding enzymes, involved in the nitrogen fixation process, are suddenly induced in the interzone II-III of the nodule under microaerobic conditions (Fischer H. 1994). Low oxygen is sensed by FixL in *S. meliloti* which activates the FixJ protein by phosphorylation upon microaerobiosis. In turn, phosphorylated FixJ induces the transcription of *nifA* and *fixK* (Batut J, 1994, Miyatake H, 2000). Both *nifA* and *fixK* genes encode transcriptional activators of *nif* and *fix* genes (Galinier A. 1994).

Root Nodule formation:

Certain bacteria can only form nodules on specific host plants, this is called host-specificity. For example, *Sinorhizobium meliloti* will form nodules on alfalfa (*Medicago*), sweet clover (*Melilotus*) and *Trigonella spp.* Recognition of the host plant and nodule formation is a multi-step process involving both host plant and the bacterium, in which so-called Nod factors play an important role. Nod factors are lipochito-oligosaccharides produced by the bacteria and are involving in the induction of the initial stages of nodulation. Bacteria *nod* genes involved in the synthesis of Nod factors are not expressed in the free-living state with the exception of *nodD,* which is expressed constitutively. Upon binding to specific flaveonoids secreted by the root of the host plant (Geothals KM. 1992), NodD activates transcription of the other *nod* genes (Fisher, 1992). Purified Nod factors induce root hair deformation at concentrations as low as 10^{-12} M (Ehrhardt DW. 1992.). Nod factors also induce the expression of certain plant genes encoding early nodulins (Van de Sande K. 1997, Heidstra R, 1997).

The bacteria in the soil are attracted to the rhizosphere by flavonoids and possibly also amino acid and dicarboxylic acids secreted by the host plants roots (Wall LG, 1991). Once in close proximity to the roots the bacteria becomes attached to the root hairs and induce a series of morphological changes which include root hair branching, deformation and curling. These changes are brought about by Nod factors, which are expressed by rhizobia in response to the flavonoids produced by the plant. The root hairs curl around and trap the rhizobia, an indentation is produced in the cell wall of the root cells and the bacteria enter the plant cell by formation of a tube-like structure known as the infection thread. The infection thread is surrounded by the plant cell wall and as the bacteria divide it elongates into the root tissue. Concomitantly, the cortical cells of the root begin to divide forming the nodule primordium towards which the infection threads grows. Rhizobia are released from the infection thread into the plant cells, but remain surrounded by a peribacteroid membrane (divided from the plant cells) and are now termed bacteroids. Cell proliferation ceases in the bacteroid state and the genes required for nitrogen fixation, such as *nif* genes which encode the nitrogenase enzyme and other components for nitrogen fixation and the *fix* genes, which form components that are specific for symbiotic nitrogen fixation, are expressed (Oke V & Long SR. 1999).

Rhizobium classification.

Nitrogen-fixing, legume root nodule bacteria are gram-negative soil bacteria classified in the alpha subdivision of Proteobacteria (Pulawska J, 2000). They are currently classified into three genera: *Rhizobium* ("fast grower"), *Bradyrhizobium* ("slower growers") and *Azorhizobium* ("stem nodulating") which so far has one characterized species that forms nodule on the stems of Sesbania.

The named species of *Rhizobium* are *R. leguminosarum, S. meliloti, R. loti , R. fredii* and *R. galegea. R. leguminosarum* has been divided into three groups to reflect the host specificity (or biovar) defined by the plasmid carried. The groups are clover (bv. Trifolii), bean (bv. Phaseoli) and pea, vetche and lentil (bv. Viciae). *S. meliloti* strains, separated into two distinct genetic groups, nodulate alfalfa (*Medicago sativa*) and related legumes (Eardly, 1990).

Using 16S ribosomal DNA comparisons techniques, it was found that *Rhizobium* and *Bradyrhizobium* are not closely related (Willems A, 1993, Liisa Koski, unpublished data, Figure 1); *Rhizoium* is closely relate to the plant tumor-inducing *Agrobacterium. Azorhizobium* is closer to *Bradyrhizobium* than to *Rhizobium*. These data are also supported by biochemical and physiological characteristics, for example, *Rhizobium* is closely related to *Agrobacterium*, their symbiotic genes are plasmid-borne, while in *Bradyrhizobium* and *Azorhizobium*, plasmids does not carry symbiotic genes. Both *Bradyrhizobium* and *Azorhizobium* can fix nitrogen asymbiotically (Reviewed by Bardin SD. PhD thesis, 1997).

The genetic structure of *S. meliloti* composes of two large extrachromosmomal replicons, referred to as megaplamids, and one chromosome of 3400kb. In *S. meliloti* SU47, the two megaplasmids have been designated pRmeSU47a (1300kb) and pRmeSU47b (1700kb). Both plasmids contain genes required for efficient nitrogen fixation; megaplasmid pRmeSU47a carries the *nod* genes and some *nif* and *fix* genes involved in nodule induction and nitrogen fixation (David M. 1987; Debelle, 1986) whereas the larger megaplasmid pRmeSU47b carries genes involved in exopolysaccharide and thiamine biosynthesis (Finan TM, 1986; Glazebrook, 1989; Zhan, 1989), high affinity phosphate transporter PhoCDET (Bardin SD, 1997),

dicarboxylic acid transporter (Finan TM. 1988; Yarosh OK. 1989, Watson RJ. 1988) as well as genes required for lactose, dulcitol, melibiose, raffinose, β-hydroxybutyrate, acetoacetate, protocatechuate and quinate utilization (Charles, 1990, 1991). Other genes are located on chromosome (3400kb) including the identified phosphate transporter system, OrfA-pit system (Bardin SD, 1997), and the possible phosphate regulator, *phoU, phoB* (Bardin SD, 1998), *phoR* and *pstSCAB* like genes, whose regulation and expression are the main subject of this study and will be addressed in detail below.

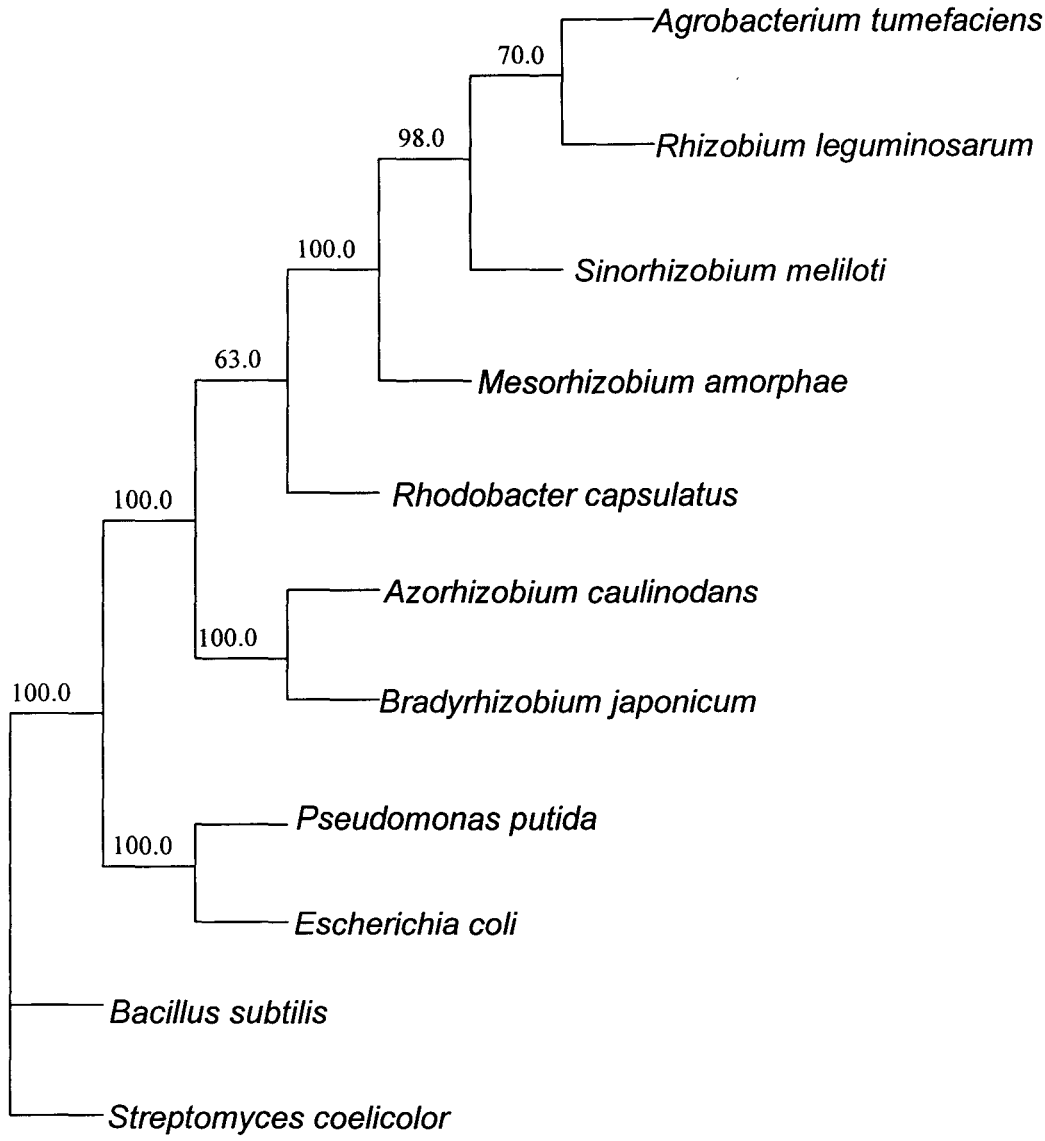


Figure.1 : Neighbor-joining phylogeny for 16S genes. The tree was constructed using the K2P model; bootstrap values (100 trials) are shown as percentages. (Constructed by Liisa Koski in August 2000, unpublished).

I-II. Phosphate uptake in microorganisms

Phosphorus is one of the most important substrates in energy metabolism and in the biosynthesis of nucleic acids and membranes. It also plays an important role in regulation of a number of enzymes. Low availability of soluble inorganic phosphate (Pi), the major assimilated form of phosphorus, is a common phenomenon in many ecosystems and often limits organism growth and survival. Consequently, assimilation, storage, and metabolism of phosphorus are highly regulated processes that immediately affect microbial growth and even survival. In the past 20 years, much work regarding microbial phosphate transport and regulation has been conducted in *Escherichia coli*.

Escherichia coli phosphate transport and regulation.

Escherichia coli possesses two major inorganic phosphate (Pi) transporters: the Phosphate Specific Transporter (Pst) and the Phosphate inorganic Transporter (Pit). In addition, it has two organophosphate transport systems (GlpT and UhpT) as well as the phosphonate transport system (PhnCDE) which is capable of taking up phosphonate as a secondary substrate (Ambudkar SV, 1986; Metcalf WW, 1990, 1991).

1. The Pst system

Pst is a high affinity low-velocity system (Km; 0.4μM and Vmax: 15.9 nmol of Pi per min per mg of protein). Its expression is inhibited when Pi is in excess and is induced 100 fold (or more) when the environmental Pi has been exhausted (Wanner BL, 1996). The Pst system is a periplasmic protein-dependent transporter belonging to the superfamily of ABC (ATPase binding cassette) type transport systems which are also called traffic ATPases. The Pst system is composed of two integral

membrane channel proteins PstA and PstC, PstB, a membrane bound ATPase (Chan, 1996) and PstS, the periplasmic phosphate-binding protein. The *pstSCAB-phoU* genes are in an operon. PhoU is a peripheral cytoplasmic protein, has no effect on Pi uptake but PhoU mutations led to growth inhibition (Steed, 1993). 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 GB, 1989). A model of phosphate transport via the Pst system is as follows: the PstS protein 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 (Webb, 1994).

2. The Pit system

The *pit* locus encodes a 499 amino acid polypeptide (Sofia HJ. 1994). Pit is a low affinity, high-velocity system (Km: 38.2 μM and Vmax: 55 nmol of Pi per min per mg of protein) and is constitutively expressed. Pit is a single-component proton motive force-driven transporter analogous to LacY (Kaback HR, 1990), also called chemiosmotic carrier. Pit utilizes $MeHPO_4$ as a substrate, a neutral metal phosphate ($MeHPO_4$) chelate, formed by Pi complexing with divalent metal ions (such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}), not inorganic phosphate (Van Veen, 1994a, b). Pit is not required for growth with Pi as sole phosphorus source and is not involved in the *pho* regulon control.

3. The organophosphate uptake systems

Like Pit, GlpT and UhpT are chemiosmotic carriers. The GlpT transport system is very similar to the UhpT transporter (Eiglmeier KW, 1987). GlpT is part of the *glp* regulon which is induced by the presence of glycerol-3-phosphate (G3P) and repressed by GlpR (Lin, 1991). Both GlpT and UhpT accumulate G3P and sugar-phosphate, respectively, in exchange for internal phosphate. Whether the GlpT and UhpT system contribute to Pi uptake under conditions of Pi limitation has not been examined. G3P can also be taken up by the Ugp system (uptake of glycerol phosphate) in response to phosphate limitation. The Ugp system is a periplasmic binding protein-dependent transporter specific for G3P and glycerol phosphoryl phosphodiesters. G3P transported by the Ugp system can only be used as phosphorous source and can not be used as carbon source (Xavier KB. 1995, Bardin SD 1997, PhD thesis review).

4. The phosphonate transport system.

Phosphonates (Pn) are organophosphorus compounds with a direct carbon-phosphorus bond. Bacteria have evolved two pathways for uptake and degradation (C-P bond fission) of phosphonates in order to use these compounds as sole phosphorus sources: phosphonatase pathway and C-P lyase pathway. *E. coli* has only the C-P lyase pathway (Wanner BL, 1990), while *Enterobacter aerogenes* carries genes for both pathways (Lee KS, 1992). The genes encoding the C-P lyase pathway components in *E. coli* are organized in a single operon made of 14 genes (*phnC* to *phnP*) (Metcalf, 1993). Pi and phosphoserine are taken up by the PhnCDE transporter as non-specific substrates (Metcalf, 1991).

5. **The *pho* regulon**

Genes induced under Pi limitation and transcriptionally regulated by the PhoB protein

constitute the phosphate (*pho*) regulon. In *Escherichia coli* more than 30 genes have been identified belonging to this regulon. These are involved in the uptake and assimilation of phosphorus compounds from environment. The *pho* regulon includes the following genes: The *pho* regulator, *phoB* and *phoR* (Makino, 1986a, 1986b); *phoA* which encodes a periplasmic alkaline phosphatase enzyme (AP) involved in the hydrolysis of organophosphates, *phoE* which encodes an outer membrane porin; and *phoH* which encodes an ATP-binding protein. Analyzing proteins synthesized during phosphorus limiting conditions revealed that 413 proteins out of the 816 studied were induced (208 proteins) or repressed (205 proteins) (Van Bogelen RA, 1996). Further analysis of these proteins will probably lead to the discovery of new genes belonging to the *pho* regulon.

6. Pi-dependent control of the *pho* regulon

The primary control of the *pho* regulon is the Pi concentration of the media. It is the external phosphate concentration, not the cytoplasmic Pi concentration, that regulates the *pho* regulon (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 JS, 1993, Appleby JL. 1996) 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 phospho-donor. It then promotes PhoB phosphorylation on an aspartate residue (Makino. 1989) via its kinase activity. Phosphorylation of PhoB enhances its binding activity to the *pho* Box, an 18 nucleotides sequence:

[5'- CT(T/G)TCATA(A/T)A(T/A)CT(T/G)TCA(C/ T)-3']

which consists 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). Inhibition of the *pho* regulon requires, a high external phosphate concentration, the PhoU protein, an intact Pst system, the phosphate specific transporter, and PhoR. PhoR is believed to dephosphorylate phospho-PhoB when the external Pi concentration increases above 4μm. Excess phosphate in the media may lead to the formation of a repression complex in which PhoR phosphatase activity is induced. When the external Pi concertration 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. Then PhoR is activated by autophosphorylation which enhanced its kinase activity and thus phosphorylated PhoB (Figure 2).

7. The Two Component Regulation Systems: PhoB and PhoR.

Two-component regulatory systems are signal transduction pathways commonly used by prokaryotes to sense and adapt to stimuli in the environment; as many as 50 different two-component systems may exist in a simple bacterium such as *E. coli*, as well as other bacteria. In addition, analogous signal transduction pathways have recently been identified in eukaryotic organisms including yeast, plants, and neurospora. These systems are characterized by a sensor kinase (often a transmembrane signaling kinase such as PhoR), which undergoes autophosphorylation on a histidine residue, and this phosphoryl group is then transferred to an aspartate residue on a response regulator protein (PhoB), which usually acts as a transcriptional activator. Sensor kinases and response regulators of

two-component regulatory systems share extensive sequence similarities to other family members, even of phylogenetically distant species. These sequence similarities probably lead to structural similarities that are responsible for cross-reactivities between sensor kinases and response regulators of different systems. This phenomenon, called "cross-talk", has been observed in vitro, and it has been implicated in complex phenotypes in vivo (Fisher SL, 1995).

In the *E coli* PhoB-PhoR two-component regulatory system, PhoB is a cytoplasmic protein composed of 229 amino acids containing at least three domains: 1- phosphorylation domain; 2- DNA-binding domain (that recognizes the *pho* Box); and 3- domain that interacts with the RNA polymerase holoenzyme. Domain 1 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) constitutively activate transcription of the *pho* regulon (Makino, 1996). PhoB specifically interacts with the σ^{70} subunit and this PhoB/σ^{70} interaction permits the RNA polymerase to enter the *pho* promoters for initiation of transcription (Makino, 1993).

The PhoR protein is composed of 431 amino acids. PhoR is anchored to the cytoplasmic membrane by two transmembrane domains (N-terminus of the protein) (Scholten M. 1993). PhoR exerts both positive and negative regulation of the *pho* regulon. The positive regulation is due to its kinase activity which has been demonstrated in vitro to phosphorylate PhoB (Makino, 1989). The negative regulation is believed to occur by dephosphorylation of PhoB. These two functions are separable from one another (Wanner BL, 1993).

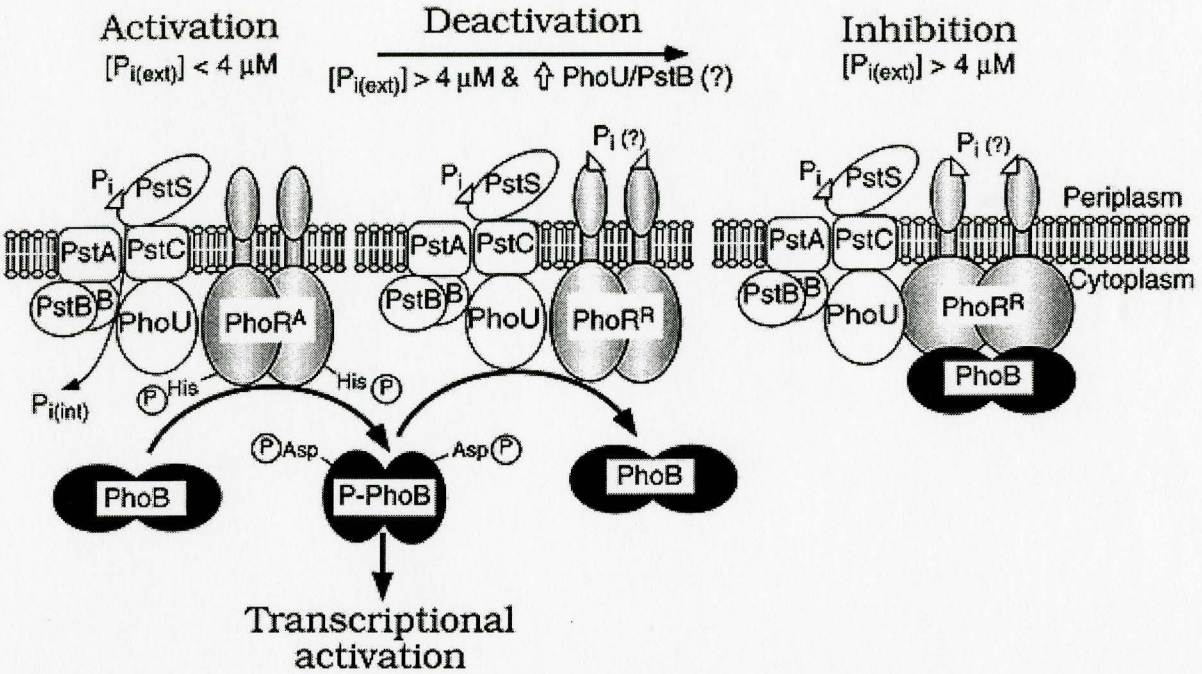


Figure 2. Model of the PstSCAB proteins involved in phosphate regulation in E. coli. PstS is a periplasmic phosphatebinding protein; phoB, response regulator; phoB-P, phospho-PhoB; $PhoR^A$, phoR activation (autophosphorylated) form; $PhoR^R$, PhoR repression(inhibition) form; PhoU, unknown function; PstA and PstC, integral membrane channel proteins of Pst transporter; PstB, traffic ATPase of transporter; small triangles, Pi binding site on the PstS and a hypothetical Pi regulatory site on PhoR. When phosphate is in excess (>4μm), the Pst complex represses the autophosphorylation of the histidine kinase PhoR. PhoU is required to inhibit the expression of the Pho regulon, but is not required for phosphate transport by the Pst system. Deletion of phoU has deleterious effects on growth, and these effects are dependent on phoB. When cells are starved for phosphate(<4μm), the Pst complex releases PhoR, which autophosphorylates and transfers the phosphate residue to PhoB. PhoB~P binds to the Pho box of pho regulon promoter and activates the transcription of most genes of the Pho regulons (in a few cases, binding of PhoB-P represses transcription). This mode was took from Dr Barry Wanner's webpage at Purdue University: (http://www.bio.purdue.edu/Bioweb/people/faculty/wanner.html).

8. Pi- independent control of the *pho* regulon (cross talk regulation).

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 which 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). So far, two Pi-independent controls of the *pho* regulon which function in the absence of PhoR have been identified: CreC (formerly PhoM), a protein kinase, like PhoR, phosphorylates PhoB as well as CreB which is a transcriptional activator that regulates the expression of unknown genes (Amemura, 1990). The other Pi-independent control requires Pta-AckA pathway (Figure 3). Mutation in the *ackA* gene (for acetate kinase) led to accumulation of acetyl phosphate. Acetyl phosphate is made from acetyl-CoA and Pi by Pta and degraded into acetate and ATP by AckA. Mutation or growth conditions leading to increased levels of acetyl phosphate synthesis, either directly (i.e. growth on pyruvate as carbon source) or indirectly, lead to activation of the *pho* regulon (Wanner BL, 1992b, Atsuo Nakata review).

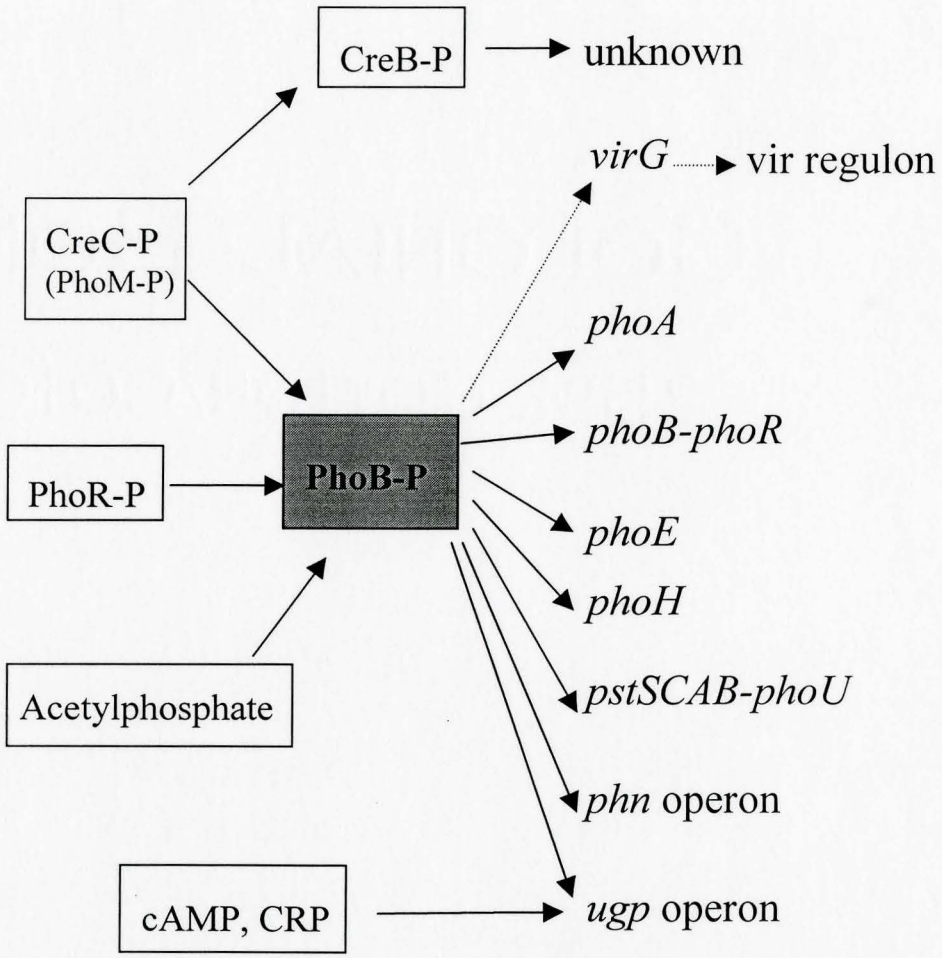


Figure. 3. Cross talk in the signal transduction related to the *Pho* regulon. The physiological (major) pathways of the signal transduction of the pho regulon are shown by bold arrows. In the wildtype strain. PhoR-P transphosphorylates PhoB and PhoB-P in turn activates transcription of the genes in the *Pho* regulon. In *A. tumefaciens,* PhoB-P activates the transcription of the *virG* gene under phosphate limitation and the elevated Level of VirG activates the transcription of the genes in the vir regulon. The ugp operon contains two kinds of promoters, one activated by phoB-P and the other activated by the CRP-cAMP complex. (reviewed by Atsuo Nakata, *Phosphate in Microorganisms.* p286).

Phosphate uptake in other micro-organisms.

In *Pseudomonas aeruginosa,*

P. aeruginosa has a high affinity (*pst*-like) phosphate transport system with an apparent Km of 0.46+/-0.1 μM and a low-affinity (*pit*-like) transporter with a Km of 12.0 +/-1.6μM (Poole, 1984). The *pst*-like system includes four open reading frames, *pstC*, *pstA*, *pstB* and *phoU* that probably constitute an operon (Nikata et al., 1996). The *pst* operon was *pho*-regulated, there is a *pho* Box preceding *pstC* and its expression is induced 25 fold in the presence of a functional PhoB protein. The most striking difference with the pst operon of *E. coli* is the absence of the *pstS* gene in *P. aeruginosa.*

In *Bacillus subtilis,*

Bacillus subtilis has the *pst* transporter (Takemaru, 1996) which includes *pstS, pstC, pstA, pstB*$_1$ and *pstB*$_2$ and likely form an operon. There are two *pstB* genes with no *phoU* gene. There are two *pho* Boxes upstream of *pstS*. The *pho* regulon of *B. subtilis* includes the structural genes for three secreted alkaline phosphatases (APase): *phoA* (encoding APaseA) is expressed primarily during phosphate starvation; *phoB* (encoding APase B) is expressed from tandem promoters either during phosphate starvation or during stage II of spore development; *phoD* (encloding APase D) is expressed during phosphate starvation. The *pho* regulon of *Bacillus subtilis* is controlled by three two-component signal-transduction systems: PhoP/PhoR, ResD/ResE, and SpoOA. ResDE and PhoPR are involved in activating the *pho* regulon, SpoA functions in repressing the *pho* regulon. PhoPR is the main regulator (response regulator and histidine kinase, respectively), *phoP* is homoguous to *phoB* in *E coli* but is named *phoP*. Other *pho* regulon include *tuaAABCDEFGH,*

pstSCABB, ResDE and *phoPR* itself. ResD (the RR) and ResE (the HK) are positive regulators of both aerobic and anaerobic respiration in *B. subtilis*. The *phoPR* operon shows low level expression during vegetative growth and is induced during phosphate limitation. On the onset of sporulation, the response regulator encoded by *spoA* represses the *pho* regulon. In *E. coli* and *B. subtilis*, *phoP, phoB* or *phoR* null mutations eliminate APase expression (Sun. 1996).

In Caulobacter,

Phosphate-starved cells undergo dramatic stalk elongation to produce stalks as much as 30 times as long as those of cells growing in phosphate-rich medium. The gene cluster of *phoR-pstCAB-phoU-phoB* were investigated with regard to the stalk elongation and phosphate starvation. *pstCAB-phoU-phoB* could be one operon. The *pstS* gene was far away from this gene cluster. PhoB is not required for stalk synthesis or for the cell cycle timing of stalk synthesis initiation. it was required for stalk elongation in response to phosphate starvation. Both *pstS* and *phoB* mutants are deficient in phosphate transport. *pst* mutants make long stalks in both high- and low-phosphate media and this phenotype is dependent on *phoB*. *pstS* transcription is dependent on *phoB*. Thus the signal transduction pathway that stimulates stalk elongation in response to phosphate starvation is mediated by the Pst proteins and the response regulator PhoB (Gonin M, 2000).

I-III. Phosphate Regulate and Other Function.

1. Phosphate and its global effects on the metabolism and gene regulation in bacteria. Limitation of inorganic phosphate leads bacterial cells to synthesize a number of proteins, many being involved in the acquisition of phosphate. However, the *pho* regulon may include genes unrelated to phosphate transport and assimulation. Also PhoB may regulate genes unrelated with phosphate transport or uptake (Wanner 1996).

2. Bacteria Metabolism and Pathogenicity. In *Escherichia coli*, phosphoribosyl diphosphate-lacking (delta *prs*) mutant strains require NAD for growth (Hove-Jensen B, 1996). *prs* strains with mutation in the *pst* system lead to NAD-independent mutants. It was well known that *pstSCAB-phoU* operon mutations lead to derepression of the *pho* regulon. This *prs* suppressor mutation (*pst* or *phoU* mutations) requires PhoB to suppress the NAD growth requirement (Hove-Jensen B, 1996). TnphoA insertions in the *E. coli* operon encoding the PstSCAB transporter and PhoU protein are pleiotropic and these mutations have an effect on the production of the surface polysaccharides of strain 5131 (Daigle F, 1995). In an *E. coli* strain pathogenic to pigs, mutation of the *phoB-phoR* lead to the loss of virulence (Lee 1989).

3. Synthesis of cell wall anionic polymer. In *Bacillus subtilis* PhoP-PhoR control the synthesis of cell wall anionic polymer, teichoic acid and teichuronic acid. Under phosphate starvation conditions, teichuronic acid is synthesized while teichoic acid synthesis is inhibited (Qi Y, 1998). A phoR-negative mutant was unable to induce the synthesis of cell wall teichuronic acid under phosphate-limited conditions (Muller JP, 1997). Besides induce the *pho* regulon, phosphate starvation also induce the sigmaB

dependent general stress regulon. These proteins provide non-growing cells with nonspecific, multiple, and protective stress resistance and are also involved in the protection of DNA, membranes, and proteins against oxidative stress and contribute to the survival of extreme environmental conditions, such as heat or osmotic stress as well as acid or alkaline shock of starved cells (Antelmann H, 2000).

4. Cell division. In *Caulobacter crescentus*, Pst proteins and the response regulator PhoB regulated the signal transduction pathway that control stalk elongation and cell division in response to phosphate starvation (Gonin M, 2000).

5. Drug efflux. In *Mycobacterium smegmatis,* Pst is a natural membrane transport system that has the ability to promote drug efflux in addition to its involvement in phosphate transport. Disruption of the *pstSCAB* operon in wild-type cells resultes in hypersensitivity to ciprofloxacin and other xenobiotics (Bhatt K, 2000).

6. Phosphate regulation and polyphosphate metabolism.

Inorganic polyphosphate (polyP) exists widely in prokaryotes. In media deficient in both Pi and amino acids, *E. coli* synthesized nucleotides ppGpp and pppGpp by either RelA or SpoT (expressed during Pi starvation) which lead to the accumulation of large amounts of polyP (Cashel, M. 1969, Spira, B. 1995). ppGpp repress many other genes, including those for ribosome synthesis, and activate (directly or indirectly) 50 or more genes responsible for coping with stress and starvation (Gentry DR. 1993, 1996). In *E coli*, potential functions of poly(P) include the following: (*i*) ATP substitute for sugar and adenylate kinases, (*ii*) a phosphate reservoir, (*iii*) a chelator for divalent cations, (*iv*) a buffer for alkaline stress , (*v*) a component in competence for DNA entry and transformation, and (*vi*) a factor in regulatory responses to stresses and nutritional deficiencies (Kornberg A, 1997). In addition, polyP is required for

swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa* (Kornberg, A. 2000). PolyP induces *E. coli rpoS* expression which supports stationary-phase resistance and survival (Shiba T. 1997). Also, polyP was required to stimulate protein degradation and adapt to amino acid starvation in *E. coli* (Kuroda A. 1999). Polyphosphate synthesis and degradation regulate the *pho* regulon. Degradation of polyphosphate during a period of phosphate limitation represses the phosphate-starvation response and this is attributed to the release of phosphate from the cell into the periplasm (Van Dien SJ, 1999).

In *P. aeruginosa*, besides involvement in different forms of bacterial motility (Kornberg, A. 2000), polyP and polyphosphate kinase (PPK) are required for biofilm development, quorum sensing and virulence of this clinically important pathogen (Rashid. 2000). polyP and PPK are also required for flagella-mediated swimming motility of other bacterial pathogens, including *Klebsiella pneumoniae, Vibrio cholerae, Salmonella typhimurium,* and *Salmonella dublin* on semisolid agar plates (Rashid, MH. 2000).

Accumulation of polyP by amino acid starvation and P_i limitation are dependent on PhoB and high levels of ppGpp. Mutants which lack PhoB do not accumulate polyP even though they develop wild-type levels of (p)ppGpp when subjected to amino acid starvation (Kornberg A, 1999). Phosphate induces the transcription of *ppk* whose product produces polyP (Geissdorfer W, 1998). Also, phosphate and amino acids deficience induce the synthesis of nucleotides ppGpp and pppGpp which lead to the accumulation of polyP (Cashel, M. 1969, Spira, B. 1995). polyP accumulation depends on several regulatory genes, *glnD* (*NtrC*), *rpoS, relA,* and *phoB* and is regulated by phosphate (Rao NN,1998, Ault-Riche D, 1998). Polyphosphate

synthesis and degradation regulate *pho* regulon (Van Dien SJ, 1999). That appear to be a feed-back regulation: 1). polyP accumulation was regulated by *pho* regulon, but polyP also regulates *pho* regulon transcription. 2). PolyP accumulation regulate RpoS expression, and RpoS regulates polyP accumulation (Figure.4).

7. IciA and DNA replication. The *E coli* IciA protein specifically inhibits the *in vitro* initiation of chromosomal DNA replication and the *iciA* gene is a member of the *pho* regulon. The phosphate depletion increases *iciA* expression. Enhanced *iciA* expression is dependent upon the PhoB protein. It was also found that the *iciA* promoter contains the *pho* box (Han JS, 1999).

8. Acid Shock. In *E. coli* the *phoB-phoR* regulatory system can sense an external acidity and positively regulate transcription of acid-inducible RNA (designated *asr* for acid shock RNA). The *asr* promoter had a region similar to the *pho* box and was demonstrated to bind PhoB protein in vitro (Normark, S. 1999).

9. Bacteria Colonization and Virulence. In *Vibrio cholerae* the *pho* regulon plays a role in its adaptation to the intestinal environment (Ketley 1999). A PhoB mutation reduced colonization ability on the rabbit intestine. In *P aeruginosa*, the expression of an extracellular heat-labile haemolysin is induced under phosphate limiting conditions and is required for virulence (Ostroff 1989). In *A. tumefaciens* and *S. typhimurium*, low phosphate levels induced synthesis of virulence factors (Winans 1990). Furthermore, mutations in entero-invasive *Escherichia coli* genes involved in phosphate acquisition gave a hyper-invasive phenotype (Sinai, 1993).

10. Phosphate regulation and plant-bacteria signal transduction. Phosphorus is required by both host plants and soil bacteria. However, low availability of soluble

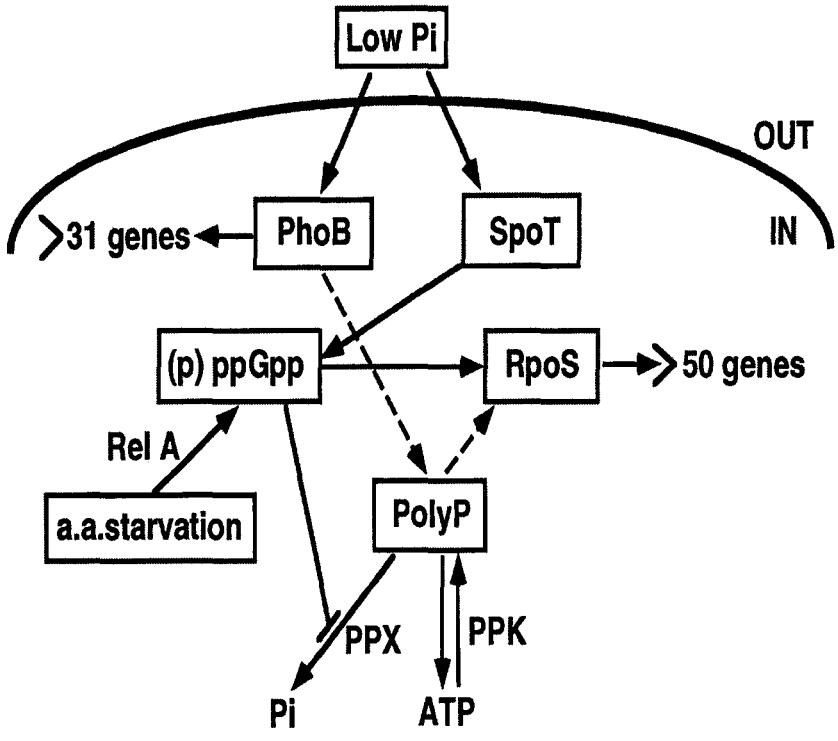


Figure. 4. Hypothetical scheme for polyP accumulation and function. PolyP accumulation depends on PhoB and (p)ppGpp synthesized either by the relA (stringent-response) pathway or by the phosphate starvation-inducible spoT pathway. The inhibition by (p)ppGpp of exopolyphosphatase PPX as one means of polyP accumulation and the dependence of rpoS expression on polyP are mentioned in the text. Dashed arrows indicate either a direct or an indirect effect on the synthesis or accumulation of the gene products. (Reviewed by Narayana N. Rao & Arthur Kornberg. J Bacteriol, April 1998)

inorganic phosphate (Pi), the assimilated form of phosphorus, is a common phenomenon in many ecosystems and often limits plant growth. Bacteria and plants utilize phosphate around roots, and therefore plants compete with microorganisms for available phosphate in the rhizosphere (Kragelund L, 1997). In addition, phosphate regulation involved in plant-*Agrobacterium tumefaciens* interaction and signal transduction.

A. tumefaciens contains about 25 *vir* genes localized on a 200-kb tumour-inducing (Ti) plasmid. The *vir* gene products direct a conjugation-like transfer of tumorigenic DNA from the bacterium to the nuclei of infected plant genome. Plant signals induce *A. tumefaciens* virulence genes through the VirA-VirG two-component regulatory system. The VirA protein is a membrane-spanning sensor molecule that possesses an autophosphorylating activity, and the VirG protein is a sequence-specific DNA-binding protein. Transcription of the *virG* gene of *A. tumefaciens* is expressed from two tandem promoters and is responsive to three stimuli: plant-released phenolic compounds (such as acetosyringone and certain monosaccharides), phosphate starvation, and acidic media (Winans SC 1990, Pan SQ, 1993). VirG protein is phosphorylated and positively regulated by the VirA protein (Jin SG, 1990, Winans SC. 1988, 1994). After phosphrylation, VirG activates the expression of other *vir* genes. The response of the virulence gene to phosphate limitation did not require the positive regulator VirG for the virulence regulon, but depended entirely on the presence of PhoB protein (Winans SC 1990, 1994). The DNA signal upstream of the virulence gene, which is targeted by the VirG protein, was also recognized by the *E. coli* PhoB protein *in vitro*. Cross-talk between the two

regulons occurred via the recognition of a DNA signal by the respective regulatory protein (Aoyama T, 1991).

I-IV. Previous work on *S. meliloti* phosphate regulation.

1. S. meliloti and Phosphate Regulation.

Phosphorus is an essential nutrient. In most soils, the concentration of soluble phosphate ranges from 0.1 - 10 μM, which is very low. To survive in low phosphate soil environment, the nitrogen fixing bacterium *S. meliloti* activates genes involved in the transport and assimilation of phosphate as well as other phosphorous compounds. *Rhizobia* and *Bradyrhizobia* take up phosphate 10 to 180 fold faster in a phosphate deficient condition than in phosphate-sufficient environment (Smart in 1984). Alkaline phosphatase (AP) activity is strongly induced in *Rhizobium* strains in response to limiting phosphate and repressed under phosphate sufficient conditions. Under phosphate-limiting conditions, they replace their membrane phospholipids by lipids not containing phosphorus. When phosphate is growth limiting, an increase in sulpholipids, ornithine lipids and the de novo synthesis of diacylglyceryl trimethylhomoserine (DGTS) lipids is observed (Geiger et al, 1999). Also, *S. meliloti phoCDET* mutants, deficient in phosphate uptake, synthesize DGTS constitutively at low or high medium phosphate concentrations, suggesting that reduced transport of phosphorus sources to the cytoplasm causes induction of DGTS biosynthesis (Geiger et al, 1999).

2. Discovery of the *ndvF* locus.

S meliloti contains two megaplasmid, pSYM and pEXO, which are 1350 and 1700kb respectively. Two overlaping defined deletions (about 150 kb) of the pEXO megaplasmid, Δ5408 and ΔF117, lead to Fix- symbiotic phenotype when the deleted strains were inoculated on alfalfa plants. These mutants induced small white nodules containing very few bacteria. The *ndvF* locus was identified after complementing the

RmF114 Fix- phenotype with pLAFR1 cosmid clone bank carrying wild type *S. meliloti* DNA (Charles, 1991). Further analysis of the *ndvF* locus revealed the *phoCDET* transport system, which encode an ABC-type high affinity phosphate transport system (Km, 0.2 μM). The *phoC* promoter contains two elements which are similar in sequence to the *pho* boxes present in *E. coli* phosphate-regulated promoters. This high-affinity transport system was induced under Pi-limiting conditions and was repressed in the presence of excess Pi (Bardin et al, 1996).

3. The *sfx* loci of *ndvF* suppressor mutations.

Following inoculation of the *ndvF* deletion mutants on the alfalfa plants, some pink Fix+ nodules were occasionally found (Oresnik, 1994). Rhizobium isolated from these nodules retained the original *ndvF* mutations but had acquired secondary mutations that fully suppressed the symbiotic deficiency caused by the *ndvF* mutations. Further analysis the suppressor locus (*sfx1*) showed that it contains two genes, *orfA* and *pit*, which form an operon transcribed in the order *orfA-pit*. These encoded a Pi transport system that is expressed in wild-type cells grown with excess Pi but repressed in cells under conditions of Pi limitation. The sfx-1 suppressor mutation arose from a single thymidine deletion in a hepta-thymidine sequence centered 54 nucleotides upstream of the *orfA* transcription start site. This mutation increased the level of *orfA-pit* transcription (Bardin et al. 1998). In *phoCDET* mutant cells, *orfA-pit* expression is repressed, but this repression is alleviated by this kind of second-site suppressor mutations. Suppression increases *orfA-pit* expression compensating for the deficiencies in phosphate assimilation and symbiosis of the *phoCDET* mutants.

Analysis of the class II suppressor mutations revealed that these mapped to the *phoU* and *phoB* regulatory genes and that disruption of these genes suppressed the

Fix- and the Pi growth phenotype of *phoCDET (ndvF)* mutants. *phoB* and *phoU* mutants was isolated from *S. meliloti*. These mutants formed normal N2-fixing nodules on the roots of alfalfa plants. Transfer of *phoB* or *phoU* insertion mutations into *phoC* mutant strains restores the ability of these mutants to form normal N2-fixing root-nodules and these strains grew like the wild type in media containing 2 mM Pi. Under Pi limiting conditions, *S. meliloti* PhoB protein activates *phoCDET* transcription and represses *orfA-pit* transcription.

4. Phosphate regulation and EPSII.

In the symbiosis of *S. meliloti* and host plant *Medicago sativa* (alfalfa), alfalfa nodule invasion by *S. meliloti* must be medicated by any one of the three symbiotically important polysaccharides: succinoglycan (EPSI), EPS II, or K antigen (also referred to as KPS) (Pellock et al, 2000). EPS (exopolysaccharides) are complex sugar polymers that are either found loosely bound to the cell as a capsule or are released into the surroundings. EPS synthesis is induced when excess carbon is available but a nutrient such as N or Pi is limiting for growth. The EPSI is the major exopolysaccharide produced by *S. meliloti*. It is an acidic heteropolysaccharide consisting of an octasaccharide repeating unit of seven glucose and one galactose molecule with succinyl, acetyl and pyruvyl modifications. Mutants unable to synthesize EPSI are defective in nodule invision and development, which demonstrates an important function for EPSI in the intermediate stages of symbiosis. A total of 22 *exo* genes identified so far has been shown to be involved in the production of EPSI, of these, 19 *exo* genes are located on pRmeSU47b while the others are located on the chromosome. Under normal culture conditions, *S. meliloti* cell produce large amounts of EPSI and no EPSII. EPSII is synthesized in low

phosphate media which can function in place of EPSI in nodule invasion (Zhan et al, 1991). 22 *exp* genes were identified for the production of EPSII which are located on pRmeSU47b, but are not closely linked to the *exo* gene cluster. The *expA, expD, expG* and *expE* promoters contain sequences with similarities to the *pho* box known as the PhoB-binding site in *E coli* and in *S. meliloti*. *S. meliloti* PhoB was required for the activation of *exp* gene expression under phosphate limitation (Becker 1997, Ruberg S, 1999).

5. Other work on phosphate regulation in *S. meliloti*.

S meliloti nonspecific acid phosphatase was cloned and characterized (Deng et al. 1998). Also *S. meliloti lon* protease gene was identified. Lon proteases mutant constitutively expressed higher levels of acid and alkaline phosphatase enzymes. This mutant was also found to form pseudonodules on alfalfa that were delayed in appearance relative to those formed by the wild-type strain, it contained few bacteroids, and it did not fix nitrogen. In addition to EPSI, the *lon* mutant also constitutively synthesized EPSII, a galactoglucan which is the second major EPS known to be produced by *S. meliloti*, but typically is expressed only under conditions of phosphate limitation (Summers et al. 2000).

Phosphotransacetylase (*pta*) and acetate kinase (*ackA*) genes in *S. meliloti* are in a operon *orfA-pta-ackA-fabI* which is inducible by phosphate starvation and controlled by PhoB. The phosphate starvation-inducible transcriptional start site upstream of *orfA* has a putative *pho* box. Mutations in either *ackA* alone or both *pta* and *ackA* did not affect the nodulation or nitrogen fixation phenotype of *S. meliloti* (Summers et al. 1999). Besides, six phosphate stress inducible (*psi*) genes in *S. meliloti* that are up-regulated in response to inorganic phosphate (Pi) starvation:

dnaK, expC, pssB, ackA, vipC, and *prkA* were cloned. PhoB was required for the *expC, ackA, vipC*, and *pssB* expression. The *prkA* was also found to be up-regulated in response to carbon starvation (Summers et al. 1998).

In addition, *S. meliloti phnG*, phn*H*, phn*I*, phn*J*, and phn*K* genes were investigated. The C-P (carbon-phosphorus) lyase enzyme encoded by this operon catalyzes the cleavage of C-P bonds in phosphonates. Disruption of the *phn* gene cluster does not prevent growth on 2-AEP, also *phnG*, phn*H*, and phn*K* gene products were not expressed in *S. meliloti* 1021 growing with 2-AEP as a phosphorus source. Moreover, their expression in *S. meliloti* was seen only during growth with particular phosphonates as sole phosphorus sources (Parker et al, 1999).

But in *S. meliloti,* before this study, we did not know, is there *phoB-phoR* two-component system which regulated phosphate uptake? Is there *pstSCAB* system and what is its function? How is *phoB* expression regulated and activated? Does phosphate regulation have any significance on symbiotic nitrogen fixation?

Chapter II. Materials and Methods.

II-I. Growth Media and Conditions:

The strains was stored in glass vials as frozen stocks at –70°C in LB broth containing 7% DMSO (dimethylsulfoxide). Frozen permanents were made by diluting 0.25 or 0.5 ml of overnight LB or LBmc cultures with an equivalent volume of LB containing 14% DMSO. Viable cells were recovered from frozen cultures by removing a few ice crestals with a sterile inoculating stick and streaking on approprate solid media.

1. LB broth contains (per liter):
   10 g tryptone (Difco Lab)
   5 g yeast extract
   5 g NaCl
   pH was adjusted by the addition of 4 ml (liquid medium) or 1 ml (solid medium) 1 N
   NaOH. LBmc (Finan 1984) was LB supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$.

2. M9 Minimal Medium (per liter of solution, 1xM9).
   5.8g disodium hydrogen phosphate.
   3g potassium dihydrogen phosphate
   0.5g sodium chloride
   1g ammonium chloride.

   The solution was autoclaved without agar or other supplement to prevent precipitation. To prepare M9 plates, sterile 2xM9 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 medium at final concentrations of 1mM, 0.25mM. 3 ug/ml and 15mM respectively. For transduction experiments, ½ LB-1/2 1xM9 agar plates were used. This medium was prepared by mixing 150ml of the LB solution containing 30g/L agar with 150ml of 1xM9 solution.

3. MOPS-buffered minimal medium.
   40 mM morpholinopropane sulfonic acid/20mM potassium hydroxide.
   20mM NH_4Cl
   2mM $MgSO_4$
   1.2mM $CaCl_2$
   100mM NaCl
   3 ug/ml biotin
   15mm glucose or succinate as carbon source.

   phosphorus source was added at the final concentration of 2mM if required. Note: the MOPS/KOH solution, the biotin and the carbon source were filter- sterilized through 0.45 μm filter and added after the rest of the ingredients were autoclaved. The glassware used were phosphate-free (rinsed in 6M HNO_3 solution).

4. Cell culture: all *E. coli* cells was grow in 37 °C. All *S. meliloti* was grow in 30 °C.

5. Growth in MOPS-buffered minimal medium:

   Cells were grown for 24 hours in LBmc, diluted the cells with LBmc to OD_{600} 0.5, then 0.5ml cells spun down, washed twice with phosphate-free MOPS medium (MOPS-P0), and 5 μl of cells resuspended in MOPS P0 was used to inoculate 5ml of MOPS medium (1/1000 dilution).

6. Water agar. Water containing15g/L agar was used to prepare plates for the germination of plant seedlings.

7. Unless otherwise indicated, all growth media was sterilized by autoclaving at 121°C, 100 Kpa for at least 15 minutes. Stock solutions of heat labile components such as antibiotics and carbon sources were sterilized by filtration through 0.45 μm filter units (Millex-HA, Millipore, Bedford MA).

8. Antibiotics were purchused from Sigma Co. or Boehringer Co. they are stored at sterile concentrated stock solutions at –20°C in H_2O, ethanol, or DMSO, filter sterilized if applicable.

Antibiotics:

For *S. meliloti*, the following concentrations (ug/ml) of antibiotics were used:

1. Ampicillin (Amp) (sodium salt) 100
2. Gentamicin sulfate (Gm) 10-20 (for lacZ-gent cassette, 40; 70 for selection of pPH1JI)
3. Oxytetracycline hydrochloride (Ot) 0.3-0.5
4. Neomycin sulfate (Nm) 100-200
5. Rifampin (Rif) 20-50
6. Spectinomycin dihydrochloride (Sp) 50-100 (200 for selection of Ω interposon)
7. Tetracycline hydrochloride (Tc) 10. (dissolve in 95% ethanol).
8. Streptomycin sulfate (Sm) 200-400 (50 for Ω interposon)
   Note: Ot and Tc were dissolved in 95% ethanol. Cm in 50% ethanol. Rif in dimethylsulfoxide (DMSO).

For *E. coli* (ug/ml):

1. Gentamicin sulfate 10
2. Ampicillin (sodium salt) 50-100
3. Chloramphenicol 10
4. Kanamycin sulfate 10-40
5. Streptomycin 50
6. Spectinomycin dihydrochloride 50
7. Tetracyclinehydrochloride 2-10.
8. X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside) used at 40 ug/ml for *E. coli* and 60 for *S. meliloti*. Purchased from Gold Biotechnology Inc. stock solution dissolved in Dimethylformamide at a concentration of 20mg/ml.
9. X-Phos (5-bromo-4-chloro-3-indolyl-B-D-phosphopyranoside) used at 40 ug/ml for *S. meliloti*. Obtained from Diagnostic Chemicals Ltd.
10. X-Gluc. (5-bromo-4-chloro-3-indolyl-beta-D- glucuronide), dissolve in N,N-Dimethylformamide (DMF). Stock solution 20mg/ml and store at -20C, Beta-glucuronidase gene (GUS) reporter. For *S. meliloti*, 40 μg/ml used.

II-II. Strains, plasmids and transposons (following page i--v).

Strains, Plasmid or Transposon	Revelent characteristics	Source, Reference or Construction
Sinorhizobium meliloti		
Rm1021	SU47 str-21	Meade 1982
Rm5000	SU47 rif-5	Finan 1984 --(68)
RmG212	RM1021 LacZ-	Glazebrook
RmF114	Δ Ω5033—5064::Tn5-233, Fix$^-$	Charles and Finan 1991
RmF921	RmG212, phoC::TnV.	Charles and Finan, 1991
Rm5408	Δ Ω5033—5007::Tn5-233, Fix$^-$	Charles and Finan 1991
RmG439	Δ G439=ndvFΔ G439, Fix$^-$	Charles and Finan 1991
RmG490	ndvF-1.7 Ω490=phoCΩ490, Fix$^-$	Charles and Finan 1991
RmG591	sfxI	Oresnik 1994
RmG672	phoCΩ490, sfx1, Fix+	S. Bardin, 1997
RmH138	ndvFΔG439, sfx1, Fix+	S. Bardin. 1997
RmH363	phoCΩ490, sfx2, Fix+	S. Bardin. 1997
RmH428	ΩphoB8::TnV	S. Bardin. 1997
RmH430	ΩphoB3::TnvV	S. Bardin. 1997
RmH615	Lac-, ΩphoB3::TnV	S. Bardin. 1998
RmH616	Lac-, ΩphoB8::TnV	S. Bardin. 1998
RmH617	Lac-, ΩphoB10::TnV	S. Bardin. 1998
RmH625	phoB8::TnV, phoCΩ490, Fix+,	S. Bardin. 1998
RmG774	sfx1-pitΩ3-10::Tn5	S. Bardin 1997
RmK262	RmG212, pTH284,	This work.
RmK263	RmG212, pTH736, phoB20::Tn5-B20, same direction,	This work.
RmK264	RmG212, pTH739, phoU21::Tn5-B20, opposite direction,	This work.
RmK265	RmG212, pTH731, phoB3::Tn5-B20, same direction,	This work.
RmK266	RmG212, pTH737, phoB18::Tn5-B20, same direction,	This work.

RmK267	RmG212, pTH736, phoU-B23::Tn5-B20, same direction,	This work.
RmK268	RmG212, pTH732, phoB-17::Tn5-B20, opposite direction,	This work.
RmK269	RmG212, pTH733, phoB-13::Tn5-B20, opposite direction,	This work.
RmK270	RmG212, pTH734, phoB-9::Tn5-B20, opposite direction,	This work.
RmK271	RmG212, pTH735, phoB-29::Tn5-B20, same direction,	This work.
RmK272	RmG212, phoU21::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK273	RmG212, phoUB23::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK274	RmG212, phoB17::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK275	RmG212, phoB9::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK276	RmG212, phoB3::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK277	RmG212, phoB20::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK278	RmG212, phoB13::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK279	RmG212, phoB29::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK280	RmG212, phoB18::Tn5-B20, pPH1JI, Sm^r Gm^r Nm^r,	This work
RmK385	RmG212, pstB::lacZ-Gm, same direction, Apase+,	This work
RmK386	RmG212, pstB::lacZ-Gm, opposite direction, Apase-,	This work
RmK387	Rm5000, pstB::lacZ-Gm, opposite direction, Apase-,	This work
RmK388	Rm5000, pstB::lacZ-Gm, same direction, Apase+,	This work
RmK389	Rm5000, pstB::ΩSp, Apase-,	This work
RmK390	Rm1021, pstB::ΩSp, Apase-,	This work
RmK391	RmG212, pstB::ΩSp, Apase-,	This work
RmK399	ϕRmK389→RmF921, pstB::ΩSp, phoC::TnV, Apase-,	This work
RmK411	RmH615, pTH733, phoB13::Tn5-B20, opposite*,	This work
RmK412	RmH615, pTH732, phoB17::Tn5-B20, opposite,	This work
RmK413	RmH615, pTH284,	This work
RmK414	RmH615, pTH736, phoB20::Tn5-B20, same*,	This work
RmK415	RmH615, pTH737, phoB18:Tn5-B20, same	This work

(opposite* means LacZ has the opposite transcriptional orientation as the fused gene. same*, means same transcriptional orientation with fused gene)

RmK421	RmG212, LacZ-Gm in the upstream of *pstA*(NotI), same,	This work
RmK423	RmG212, LacZ-Gm in the upstream of *pstA*(NotI), opposite,	This work
RmK426	ϕRmH615→RmK385, pstB::lacZ-Gm, phoB::Tnv, same.	This work.
RmK428	ϕRmH615→RmK386, pstB::lacZ-Gm, phoB::Tnv, opposite.	This work.
RmK430	RmG212, pstA::lacZ-Gm (SphI), same.	This work.
RmK432	RmG212, pstA::lacZ-Gm (SphI), opposite.	This work.
RmK433	RmG212, phoR::lacZ-Gm (SphI), same.	This work.
RmK435	RmG212, phoR::lacZ-Gm (SphI), opposite.	This work.

Escherichia. coli

MT607	MM294A recA56	T M Finan 1986--70
MT609	thyA36 polA1 ap-r	T. M Finan---67
MT616	MT607 (pRK600), mobilizer	T. M. Finan 1986—70
MT620	MT607 rif-20	T. M. Finan
DH5α	ϕ80dlacZ ΔM15	GIBCO BRL.
G312	MT607 Ω5::Tn5-B20	B. Driscoll and T. M. Finan

Transposons:

Tn5	Nm^r / Km^r, Sm^r (pRK602)	Berg 1987
TnV	Tn5 containing pSC101 *oriV*, Nm^r / Km^r	Furuichi 1985
Tn5-132	Ot^r (pRK604)	Berg 1987
Tn5-B20	Tn5 with lacZ transcriptional fusion, Nm^r / Km^r	Simon 1989

Phage

M12	*S. meliloti* transducing phage	Finan 1984

Plasmids

pAB1002	pUC derivatives with LacZ-Gm cassette, Amp^r,	A. Becker, 1995.
pAB2001	pUC derivatives with LacZ-Gm cassette, Amp^r,	A. Becker, 1995.
pBBR-5	Clone vector, Gm^r, rep, mob.	K. M. Peterson, 1995.
pK18/19GIImob.	Suicide vector, gusA, Km-Nm^r, mob.	L. Lelpi, 1999
pJB3J1	R68.45 derivative, Tc^r, Km^s	Lab collections.
pRK600	pRK2013 npt::Tn9, Cm^r, Nm-Km^s.	Finan, 1986
pRK607	pRK2013::Tn5-233, Nm-Km^r Gm^r Sp^r	De Vos. 1986
pPH1JI	IncP broad host range plasmid, Gm^r Sp^r Cm^r	Beringer 1978
pHP45Ω	pBR322 Ap^r containing Sm^r Sp^r Ω fragment	Prentki 1984
pLAFR1	broad host range cosmid vector, Tc^r	Friedman 1982
pRK7813	broad host range vector, poly-cloning site, Tc^r	Jones 1978
pUC119, 118,	pUC18, 19 containing intergenic (IG) region of M13.	Messing 1987
pTH282	pLAFRI cosmid clone restoring APase of RmH399, #3,	B. Schoeman
pTH284	pLAFRI cosmid clone restoring APase of RmH399, #7,	B. Schoeman
pTH286	pLAFRI cosmid clone restoring APase of RmH399, #11,	B. Schoeman
pTH292	SalI ligation of RmH399 (pho-10::Tnv).	S. Bardin. 1997
pTH301	pUC118 with 2.7kb HindIII/SalI fragment from pTH292, *pstB,*	S. Bardin. 1997
pTH583	pUC119 with 17kb EcoRI fragment from pTH284, *phoUB*,	This work
pTH609	pUC119 with 4.5kb HindIII from pTH283.	This work.
pTH611	pUC119 with 10kb HindIII from pTH284 containing *phoB*.	This work.
pTH613	pUC119 with 6.5kb HindIII from pTH286`	This work.
pTH621	pLAFRI, pTH284 with 6.5kb BamHI deletion	This work
pTH625	pUC119 with 9kb BamHI/EcoRI fragment containing *phoUB*,	This work
pTH626	pBBR-5 with 9kb BamHI/EcoRI fragmentfrom pTH625, *phoUB*,	This work
pTH628	pBBR-5 with 6.5kb KpnI fragment, complement phoUB mutation,	This work.
PTH658	pUC119 with 2.7kb HindIII/EcoRI from pTH628, *pstAB*,	This work

pTH659	pK18GIImob with 2.7kb HindIII/EcoRI from pTH628, *pstAB*,	This work
pTH660	pK19GIImob with 2.7kb HinDIII/EcoRI from pTH628, *pstAB*.	This work
pTH663	pK18GIImob, 2.7kb pstAB, pstB::lacZ-Gm (SmaI), opposite,	This work
pTH664	pK18GIImob, 2.7kb pstAB, pstB::lacZ-Gm (SmaI), same,	This work
pTH665	pK18GIImob, 2.7kb pstAB, pstB::Ω insertion(SmaI),	This work
pTH691	pUC119 with 7.5kb HindIII fragment from #7 R-prime plasmid,	This work
pTH693	pTH691 with 2.4kb and 0.8kb NcoI deletion, *pstS*,	This work
pTH697	pK19GIImob, 2.5kb HindIII/EcoRI, phoR,	This work.
pTH704	pTH697, phoR::LacZ-Gm (SphI), opposite,	This work
pPH705	pTH697, phoR::LacZ-Gm (SphI), same,	This work
pTH707	pTH659, pstA::LacZ-Gm (SphI), opposite,	This work.
pTH709	pTH659, pstA::LacZ-Gm (SphI), same,	This work.
pTH711	pTH659::LacZ-Gm (NotI), upstraem of pstA, same,	This work.
pTH713	pTH659::LacZ-Gm (NotI), upstream of pstA, opposite,	This work.
pTH731	pTH284, phoB3::Tn5-B20, blue on X-Gal,	This work.
pTH732	pTH284, phoB17::Tn5-B20, white on X-Gal,	This work.
pTH733	pTH284, phoB13::Tn5-B20, white on X-Gal,	This work.
pTH734	pTH284, phoB9::Tn5-B20, white on X-Gal,	This work.
pTH735	pTH284, phoB29::Tn5-B20, blue on X-Gal,	This work.
pTH736	pTH284, phoB20::Tn5-B20, blue on X-Gal,	This work.
pTH737	pTH284, phoB18::Tn5-B20, blue on X-Gal,	This work.
pTH738	pTH284, phoUB23::Tn5-B20, blue on X-Gal,	This work.
pTH739	pTH284, phoU21::Tn5-B20, white on X-Gal,	This work.

II-III. Methods and Techniques.

1. Preparation of competent cells (Calcium Chloride method) and transformation.

This method was used to transfer plasmid DNA into competent *E.coli* DH5α cells. Overnight saturated DH5α culture was used to inoculate 100ml of LB broth. The cells were grown to an OD_{600} of 0.4-0.6 and centrifuged for 5 min at 4500rpm, 4°C. The subsequent steps were performed on ice. The pellet of cells was resuspended in 50ml of an ice cold solution of 50mM $CaCl_2$ and 20mM potassium acetate (pH 6.2) and left on ice for one hour. After 5 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 or 5μl of plasmid DNA. The mixture was left on ice for an hour, heat shocked for 1min at 42°C and put back on ice for 1 min. 0.5ml of LB broth was added to the cells and the culture was grown for one hour before being pelleted, plated on to LB plates containing suitable antibiotics to select for the plasmid. Colonies appeared after 12 hours growth at 37°C.

2. Preparation of ϕM12 transduction Lysates.

To prepare a ϕM12 phage lysate from log phase LBmc *S. meliloti* cultures (the phage requires calcium for infection), 5ml of an overnight culture grown in LBmc was diluted to an OD_{675} of 0.4-0.5 in the same media. 50ul of ϕM12 phage stock propagated on RM1021, with a titre of about 10^{10}-10^{11} PFU/ml, was added to 2.5 or 5ml of above S. meliloti culture. This was incubated on a 30°C rotating wheel for 12

hours with areation to allow complete lysis of the cells (clearing of the culture). A few drops (200μl) of $CHCl_3$ were added to kill any viable cells that remained. 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. Lysates were then stored at 4° in 13x100mm screwcap test tubes. 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.

To determine the phage titer, the lysate was diluted in 1:10 series in LBmc. Mix 0.1 ml of an appropriate phage lysate dilution in LBmc was added to 0.1 ml of an overnight culture of Rm1021 in LBmc. After 15 minutes at room temperature to allow adsorption of the phage particles to the bacterial cell surface, 2.5 ml of LBmc 0.5% agar, cooled to about 50°C, was added. The mixture was immediately poured on the surface of an LBmc agar plate and after solidification of the soft agar, incubated overnight at 30°. The titre, in PFU (phage forming units per ml), was calculated by multiplying the number of plaques arising in the bacterial lawn after overnight incubation by the dilution factor and dividing by 0.1.

3. Generalized transduction.

Generalized transduction involves the transfer of genomic DNA fragment from one strain to another via a transducing phage that is able to package random fragments of host DNA. The phage used for transduction in *S. meliloti* is ΦM12 (Finan, 1984). 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 (OD_{675} ~1). This gave a multiplicity 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 2.5-3ml saline. The pellet of cells was resuspended in 1ml saline. 0.1ml was plated on ½ LB-1/2 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.

4. 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. This 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), where c represents the cotransduction frequency, d the distance between markers (kb) and L the lengh of DNA in the transducing particle (for ϕM12 (160kb); Charles and Finan, 1990; Finan, 1984, Figure 5).

5. Conjugal mating

The transfer of a foreign plasmid DNA into *S. meliloti* cell required mating between the plasmid donor strain (*E. coli*) and the recipient cell. Mating of mobilizable plasmids was facilitated by the presence of an *E. coli* strain MT 616, 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 *Sinohizobium* recipient strain. The *E. coli*

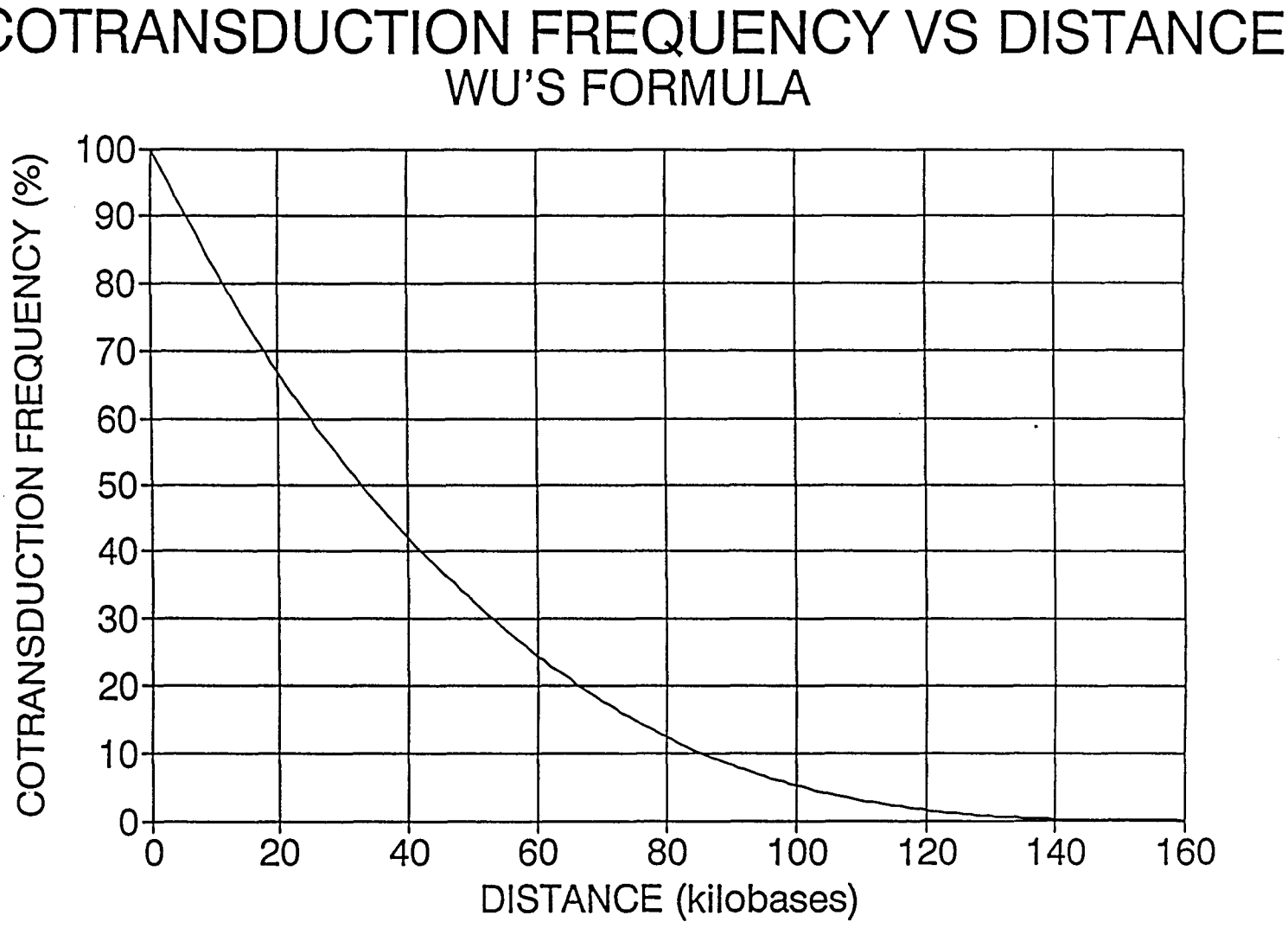


Figure 5. ϕM12 (160kb) Mediated Cotransduction Frequency VS Distance, by Charles TC and TM Finan, 1990.

culture was spun down and resuspended in LB media to eliminate of the antibiotics. 20μl of each culture was mixed and the resulting 60μl was spotted on an LB plate. As controls, 20μl of each strain culture was spotted on their own on an LB plate. After 16hours incubation at 30°C, the spots were resuspended in 1ml saline (0.85% sodium chloride) and 100μl 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°C.

6. Homogenotization (marker exchange using replicable vector).

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 *Sinorhizobium* 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 IncP 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 pRK7813 plasmid was confirmed by the Tc sensitive 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).

7. Homogenotization (recombination using suicide vector).

First choose suitable suicide vector which can not replicate in *S. meliloti*. In this study, pk18/19GIImob suicide vector was used. This vector contains the Km/Nm resistance gene and *gusA* reporter gene (Becker 1998). Subclone the gene fragment of interest from *S. meliloti* into the multiple clone site of this vector and ligate and transform into *E. coli* DH5α cells. Select on LB with Km_{30} and X-Gal_{40} for white clones and purify the white clones for 2-3 times, then plasmid DNA was extracted from these white clones. Then insert the *lacZ-Gm* or some other insertion cassette into the suitable restriction site of the subcloned gene fragment in the suitable directions (opposite or same direction with the cloned *S. meliloti* gene), then select on LB with Km_{20} and the suitable antibiotics for the insertion marker. At this stage, for lacZ-Gm cassette insertion, if the interested gene fragment contain the complete promoter, X-Gal_{40} was added into the selecting plates. This will allow to tell the cassette insertion directions for blue and white because the promoter of the interested gene will cause the expression of *lacZ* gene (only in *Sinorhizobium*). Also for the last stage, after recombination into chromosome, the direction was also can be checked in this way. After the cassette insertion and the desired clones were purified. To recombine the insertion into *S. meliloti* chromosome, *E coli* strains carrying the above plasmid (suicide vector with subcloned gene and insertion) was mated with *S. meliloti* strains with the helper strain of MT616 (pRK600, mob). Then select on LB with Sm_{400} and antibiotics for the insertion marker (Gm_{40} in this case) for single cross-over

(recombination of the whole plasmid into the chromosome). Patch 200-400 of the single cross-over colonies on LB plate with Nm_{200} ans Sm_{400} and LB with Gm_{40} and Sm_{400} for selecting clones lossing the Nm_{200} marker. The Nm sensitive clones were presumed to arise via a double cross-over. This was double check by streaking the strains into LB with X-$Gluc_{40}$, the double cross-over should be white and the single cross over should be blue (because the GusA gene in the vector).

8. Tn5-B20 mutagensis of Plasmid DNA

Random Tn5-B20 insertion on a IncP plasmid carrying a fragment of interest were generated by a two step procedure. The plasmid was first introduced into *E. coli* G312 which carries Tn50-B20 on the chromosome by selecting on LB with Km20 and Tc10. In the second stage of the procedure, the Km and Tc resistant plasmid was moved from the G312 into MT609, an E coli Sp^r *polA* strain by selection on medium contain Sp100, Km20 and Tc2. Usually, 10-20 colonies were randomly picked up from the first mating as donor to ensure that we obtained enough independent random Tn5-B20 insertions. Tn5-B20 insertion that lie within a region that can complement a specific mutant phenotype were sorted by transfer into the corresponding *S meliloti* recipient strain following by testing the transconjugant for complementation of the mutant phenotype. The insertion which can not complement the mutation of *S. meliloti* phenotype indicate that the plasmid gene was mutated. Finally the location of the insertion was determined following transfer of the plasmid into *E coli* MT607 by counter selection against *Rhizobium* at 37°C overnight inocubate. Plasmid DNA was isolated from MT607 and analyzed by restriction mapping using appropriate enzymes. Often, plasmid DNA isolated from MT607 was used to transfer *E. coli DH5α,* which give better yields and quality of plasmid DNA.

9. Random Tn5-B20 mutagenesis of the *S. meliloti* genome.

Transposon Tn5 is 5.7kb in size and carries genes encoding resistance for Nm/Km, neomycin and Sm which are flanked by inverted repeats (IS50 elements). Tn5-B20 was a Tn5 derivative with *lacZ* fusion into Tn5 transposon. They can transpose from one replicon to another at a frequency of about 10^{-4} to 10^{-5} per recipient. Random mutagenesis of *S. meliloti* genomic DNA was performed by introducing a suicide plasmid (plasmid that cannot replicate in *Rhizobium*) carrying Tn5-B20 from an *E.coli* strain and selecting for the Nm^r recombinants. Spin down and wash overnight culture of *S. meliloti* RmG212 and *E. coli* G351 (pRK600:Tn5-B20) in LB and mate them in the same volume. For best results, the E. coli cells are general subcultured for about four hours prior to the mating). Remove the mating spot and each control and resuspend in 0.85% NaCl. 10^{-1}—10^{-6} dilution. Spread 1x0.1ml of the 10-1 and 10-2 dilutions of the mating mixture and 0.1ml from each undiluted contreol tube onto selective medium ($LB/Nm_{200}/Sm_{400}$/X-$phos_{40}$). Spread 1x0.1ml of the 10^{-6} and 10^{-5} dilutions of the mating mixture onto each of LB and LB with Sm_{400}. Inocubate the LB plates at 37°C and all the other plates at 30°C. The white clones are mutations occur at the phosphate regulation or uptaking pathway. purify the Nm^r transconjugants 2-3 times. To further locate each Tn5-B20 insertion. Need replace the Tn5-B20 transposon with TnV which contain *oriV*.

10. Plasmid DNA preparation.

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 (3.0ml of cells), 100μl of solution 1 (50mM glucose, 25 mM Tris –HCl pH8, 10mM EDTA pH 8), 200μl Solution 2 (0.2N NaOH, 1% SDS) and 150μl Solution 3

(60ml 5M K-acetate, 11.5ml glacial acetic acid, 28.5ml H_2O were used. Large scale preps were further purified using the LiCl; PEG/NaCl precipitation procedure described in Sambrook et al., (1989; pp. 1.41).

11. ***S. meliloti genomic*** **DNA preparation.**

5ml LBmc overnight culture was centrifuged at room temperature 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, 150mM NaCl) and were resuspended in 2.5ml of $T_{10}E_{25}$ to which 250µl lysozyme (2mg/ml in $T_{10}E_{25}$) was added. The mixture was incubated 15 min at 37°C. 300µl 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 autolysis, 10% sarkosyl was added (The solution was kept at –20°C), and incubated at 37°C 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 volume 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 300µl $T_{10}E_{1}$. 1µl of 100µg/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 volume of 3M NH_4Ac and 2 volume 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 200µl $T_{10}E_{1}$. 5µl was checked on an agarose gel.

12. DNA restriction digestion and ligation.

Restriction of plasmid or total DNA was done in a volume of 20-50ul containing between 100-500ng DNA at the suitable temperature for 1-4 hours. Restriction reaction contain 1 units enzyme and appropriate buffer. For double digestion, if the two enzyme can be used same buffer, it is perfect, also one of the enzyme has above 40% activity. The digestion can be performed. Otherwise, two separate digestions are needed. After the first digestion, add ddH_2O and 5M NaCl to the reaction mixture (50 ul mixture + 1 ul of 5M NaCl). Then add 95% ethanol to make the final concentration to 75%. (about 2 volume of ethanol was added). On ice 20 minutes. 12500 rpm for at least 10 minutes. The pellet was washed by 100% ethanol (500μl) and dry. Then suspend in ddH_2O and do the second digestion as usual. Then run on 0.8% agarose gel in TAE buffer (40mM Tris-HCL, 20mM sodium acetate, 2 mM EDTA, pH 8 with glacial acetic acid). Fragment were stained with Ethidium bromide (5 μg/100ml staining volume) for about 10 minutes, briefly destained with ddH_2O if necessary and visualized using a UV light source and photograph. Ligation was usually carried out in 10 μl volumes. DNA to be inserted and vector DNA in a ratio of about 2:1. Ligations were carried out overnight or for more than 5 hours at 16 degree or just in room temperature (25°C).

13. DNA gel purification from low melting point agarose gels.

1. plasmid DNA was digested for 1-2 hour and run on 0.8% agarose gel, EtBr stained.

2. cut the desired band under the UV light.

3. weigh the fragment and transfer to eppendoff tubes.

4. 5 volume of 50 mM Tris-HCl pH8, 20 mM EDTA was added

5. heat to 68 °C for 20 minutes.

6. extract with equal volume of phenol, twice with chloroform/isoamylacohol (24:1).

7. ethanol precipitated.

A commericial available DNA purification kit (QIAGEN Co.) was also used to get good qualify DNA.

14. Plant growth test (method modified by Brian Driscoll, PhD thesis).

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.

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 $CaHPO_4$, 0.2g/K_2HPO_4 ,0,2g/l $MgSO_4.7H_2O$, 0.2g/l NaCl, 0.1g/l $FeCl_3$, and 1ml/l trace minerals. The trace minerals solution was composed of 1g/l H_3BO_3 , 1g/l $ZnSO_4.7H_2O$, 0.5g/l $CuSO_4.5H_2O$, 0.5g/l $MnCl_2.4H_2O$, 1g/l $NaMoO_4.2H_2O$, 10g/l EDTA, 2g/l NaFe-EDTA and 0.4g/l Biotin). The whole apparatus autoclaved for 2 hours.

Sterilization of the seeds, Alfafa seeds (*Medicago sativa* cultivar Iroquois; 100 seeds weighting 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).

Inoculation of the seedings, Ten germinated seedings 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 *S. meliloti* saturated culture (OD_{600} 1 to 1.5) diluted to 10ml of sterile water ($1x10^7$-$1x10^8$ cells) were used to inoculate a Leonard jar assembly containing emerging seedings. Three jars were inoculated with the same bacterial strain (total of 30 seedings). The plants were grown up to one month and were watered as needed with sterile distilled water.

Acetylene reduction assay (modified by Sylvie D. Bardin, PhD thesis, p75—76).

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 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-Pakard 5890 gas chromatograph (GC; air 34psi, H_2 12psl N 65psi). The ethylene peaks were integrated using the HP3365 Series 2 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 (P_r), 1 mole of gas has a volume of 22.4l (=R), 1ml of gas then contains 1/2.24 $x10^{-4}$ moles. 1ml of 506ppm ethylene (506 part/10^6 =5.06 $x10^{-4}$) will have $5.06x10^{-4}/2.24x10^4$ =$2.26x10^{-8}$ moles and 0.2ml of 506ppm ethylene, $2.26x10^{-8}/5$=4.52 nmol. By relating the unit peak area, when 0.2ml of a 505ppm ethylene standard was injected to the GC, to the number of mole, the number of mole per unit area can be determined (Znmol/unit). The acetylene reduction per plant per hour of the sample can then be calculated from the general formula (ARA=):

$$\frac{\text{Area units of sample x Znmol/unit x 150(0.2ml is 1/150 the bottle volume)}}{\text{3 plants x 0.25 hours (=15 min)}}$$

Determination of the plant dry weight, The plant shoots from each jar were cut at the root-shoot junction. All plant tops from each pot were put together in a paper bag (8-11 plant tops per bag) and dried k in a 70°C oven for at least one week. The dry weight (in mg per plant) was determined by weighing the dried plants of each bag and dividing by the number of shoots.

15. β-Galactosidase assay (Sylvie D. Bardin, PhD thesis, p78)

0.5ml of overnight grown cells (for which OD_{600} was measured) was mixed with 0.5ml of Z Buffer. Z Buffer was adjusted to pH 7, not autoclaved and contained, per litre: 16.1g $Na_2HPO_4.7H_2O$ (60mM), 0.75g KCl anhydrous (10mM), 0.246g $MgSO_4.7HO$ (1mM) and 2.7ml of β-mercaptoethanol (0.05M) added just before use), add 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_2CO_3. The tubes were centrifuged 5 min at 12,000rpm and the optical density at 420 nm was measured.

the β-galactosidase activity, in miller units was calculated using the following formula:

$$(1000.\ OD_{420}) /\ (OD_{600}.\ \text{Time}.\ V)$$

T indicate reaction time (min) and V indicate volume of culture used (ml).

16. Alkaline phosphatase assay.

1. Grow bacteria cell in appropriate media overnight (>16 hours), and if necessary with suitable antibiotics.

2. At the same time, appropriate negative or positive control cultures, including the wildtype strain were grown.
3. The cells were centirfuged and washed if necessary, for phosphate stavation test, the cells should be washed by the minimal media without phosphate.
4. Resuspended the cell pellet in 1M Tris-HCl (pH8) buffer to OD_{600} of about 0.1. Measure the OD_{600} of each test tubes and record.
5. 3ml of the diluted cells were equilibrated at 30 degree for 5 minutes.
6. The reaction was started by adding 500 ul of 4 mg/ml nitrophenyl phosphate (NPP) and record the starting time exactly.
7. After the color become yellow (or after 1 hour), the reaction was stopped by adding 600 ul of 1 M phosphate solution (K_2HPO_4), and the precise time was noted.
8. The tubes were centrifuged 5 minutes at 12000rpm.
9. The superant was transferred to the spectrophotometer tubes to measure OD_{420}.

The alkaline phosphatase activity was calculated using the following forula:

$$(1000. OD_{420}) / (OD_{600}. Time).$$

T: reaction time (min).

17. R-prime techniques.

Construction of R-prime plasmids was based on the ability of R68.45 to mobilize the DNA of the host strain at high frequency (Riess et al., 1980). First, the R68.45 derivative pJB3JI (which is Tc10 resistance) was directly transferred into host strain by overnight mating at 30 °C. The host DNA fragment in which you are interested in must contain a select marker (for example, antibiotics resistance). Transfer pJB3J1 into the donor strain. This resulting strain was used as donor in

conjugational mating with recipient strain E. coli MT620, which is Rif resistance (Rif20). Transconjugants were selected on LB with Rifampicin (20 μg/ml -1) and the suitable antibiotics (for Tn5-B20, Km20ug/ml was used). Transconjugants were grown overnight for plasmid DNA isolation. R-Prime plasmids carrying host strain DNA was checked by size comparsion on agarose gels and digestion with suitable restriction enzymes.

Chapter III. Results

1. *S. meliloti phoB* expression is not regulated by phosphate nor auto-regulated.

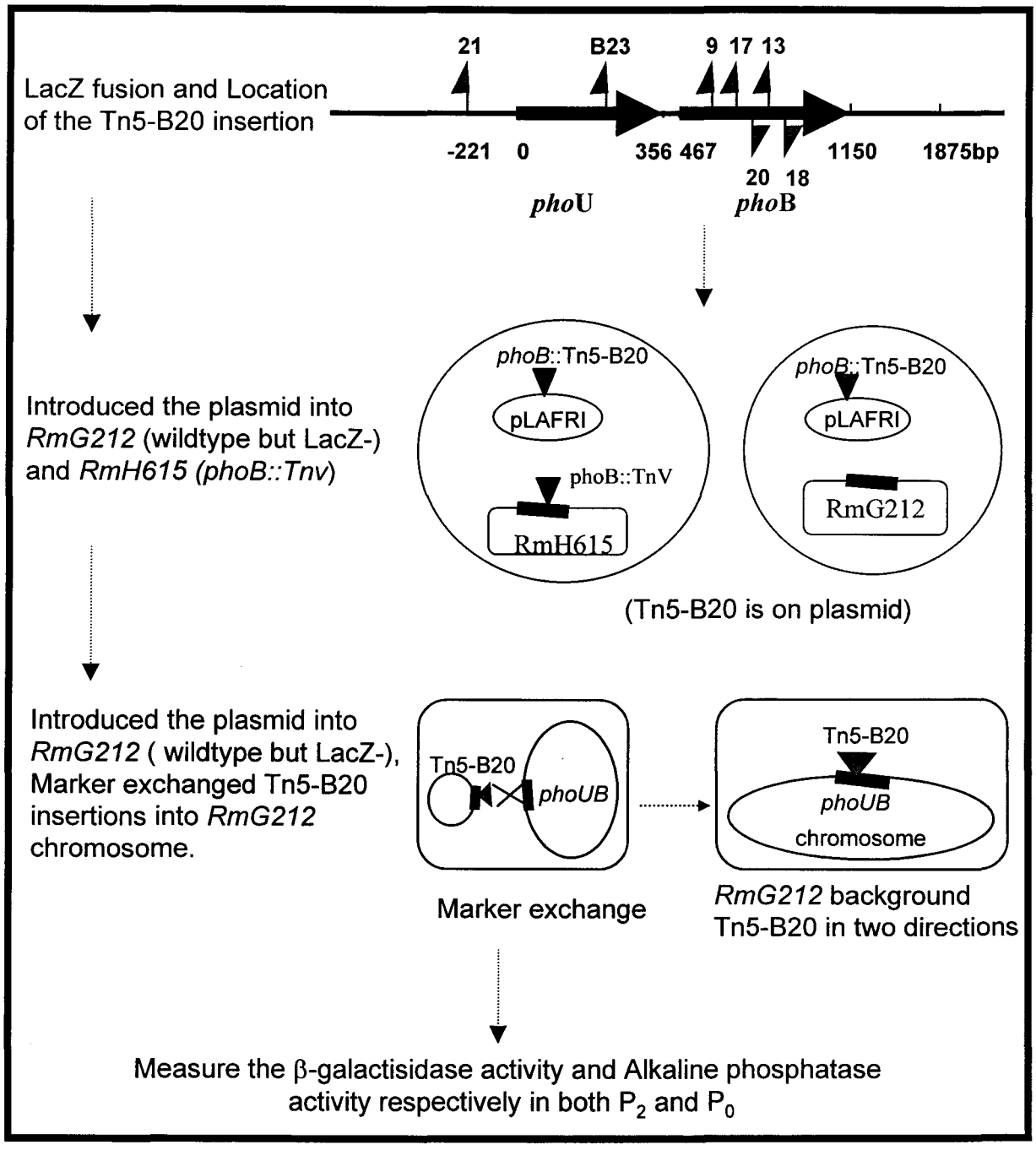
In *E. coli*, *phoB–phoR* genes form one operon and are also components of the *pho* regulon. In the promoter region of *phoB*, there is a *pho* box. *PhoB* expression is regulated by media phosphate concentration and is autoregulated (self-regulated). At low Pi concentration, PhoR is autophosphorylated and phosphorylates PhoB. Phosphorylated PhoB binds to the *pho* Box and activates transcription of the genes of the *pho* regulon. At high Pi concentration or phosphate excess, PhoR dephosphorylates PhoB-P, PhoB no longer binds to the *pho* box and the *pho* regulon is not activated. Previous research found that *S. meliloti* has one high affinity ABC type phosphate transport system, *phoCDET*, which is transcribed in low phosphate conditions and is required for symbiotic nitrogen fixation, one low affinity phosphate transport system *orfA-pit* which is transcribed in high phosphate conditions. PhoB is required for *phoCDET* expression but repress *orfA-pit* expression in free-living conditions. PhoB mutants form Fix+ nodules. It is unkonwn if *phoB* expression is regulated by phosphate concentration and auto-regulated.

The strategy we use to address this question was outlined in Figure 6.

1. Isolate cosmid that contains the *S. meliloti phoUB* gene region,

S. meliloti cosmid library was made by *EcoRI* partial digested *S. meliloti* genome DNA and cloned into pLAFRI vector (Freidman et al 1981). In previous work cosmid clones pTH283, pTH284. pTH286 which complemented the alkaline phosphatase negative phenotype of *S. meliloti phoB* mutants were isolated (Bardin et al 1996).

Figure 6. **Strategy for studying if *S. meliloti phoB* expression is regulated by phosphate? And if auto-regulated ?**



2. *LacZ* gene fusions in the *phoUB* genes were generated by isolating Tn5-B20 insertions in pTH284. Plasmid pTH284 can complement *phoB* mutations. Transposon Tn5-B20 was randomly inserted in pTH284, and insertions which failed to complement *phoUB* mutants were identified. Plasmids DNA were prepared and the insertion site was located by DNA sequencing using the *LacZ* primer (-40 universal primer):

5'----GTTTTCCCAGTCACGAC----3'

The exact location of Tn5-B20 insertions and the direction of *lacZ* transcription are indicated in Figure 7:

3. The Tn5-B20 insertions were marker exchanged into the RmG212 (wildtype and LacZ-) chromosome. The procedure for marker exchange is given in the chapter II.

4. The above mutants were grown in MOPS buffered minimal medium with 2 mM phosphate (K_2HPO_4) (P_2) and without phosphate sources (P_0), with RmG212 as control. After 16 hours growth, the β-galactosidase and alkaline phosphatase activities were measured. The results are given in Figure 8. Insertions #18 and #20 are orientated such that *lacZ* and *phoB* are transcribed in the same direction, insertion #17 *lacZ* fusion is transcribed in the opposite orientation to *phoB*. Similar β-galactisidase activities were detected in P_0 and P_2 grown cells. We conclude that phoB expression is not regulated by phospahte concentration. In this experiment, we could not measure the alkaline phosphatase activity since PhoB is required for *phoA* expression. But we are sure that the experiment is conducted in the same P_0 and P_2 conditions, because the same MOPS buffered medium and same method for washing the cells was used for this experiment and the next experiment in which we investigated whether *phoB* is auto-regulated (Figure 8). From Figure 8, we can tell

that the cells are phosphate starved because the high APase activity was detected for the wildtype strain RmG212 grown in P_o.

To investigated whether *phoB* expression is auto-regulated, plasmid pTH284 carrying *phoB*::Tn5-B20 insertions was transferred into RmG212 (wildtype and *LacZ*-) and RmH615 (*phoB*::Tnv mutant). After 16 hours growth in MOPS P_o and P_2, the β-galactisidase and alkaline phosphatase activities were measured and the results are *given in Figure 9.* In the background of RmH615 (*phoB*::TnV) and RmG212 (wildtype, *lacZ*-), #18 and #20 *phoB-lacZ* fusions has same expression level in both P_0 and P_2. In general, the *phoB-lacZ* expression levels in Figure 9 were higher than in Figure 8 presumably because the *phoB-lacZ* fusions were plasmid located, but in chromosome, *phoB* is single copy or low copy. So *phoB* expression is independent of *phoB* itself. In both the wildtype and the *phoB* mutant background, *phoB* was expressed at samiliar levels. This result that *phoB* expression is not regulated by phosphate concentration nor auto-regulated is totally different from the situation in *E. coli*. In *E coli*, *phoB* expression is self-regulated and also regulated by phosphate concentration. So, in *S meliloti*, there must be some other factor which activate *phoB* expression under phosphate starvation condition.

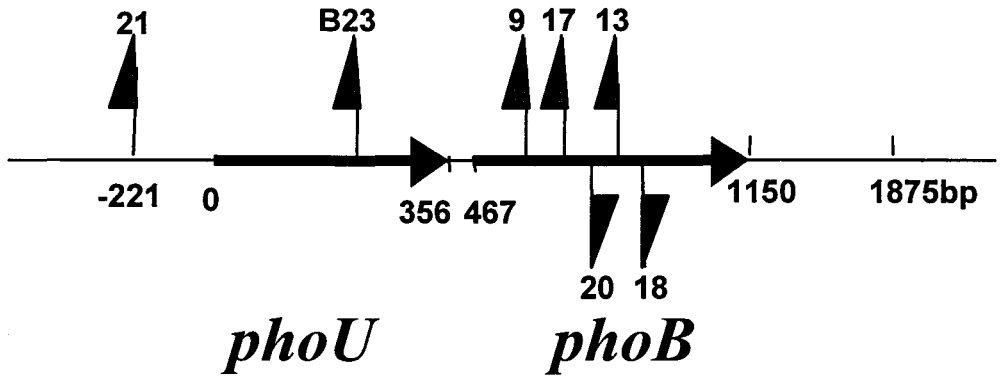


Figure 7. Location of the Tn5-B20 insertion in plasmid pTH284. Cosmid pTH284 contains the *S. meliloti phoUB* gene region. *LacZ* gene fusions in the *phoUB* genes were generated by isolating Tn5-B20 random insertions in pTH284. Flags indicate the direction of transcription of *lacZ*.

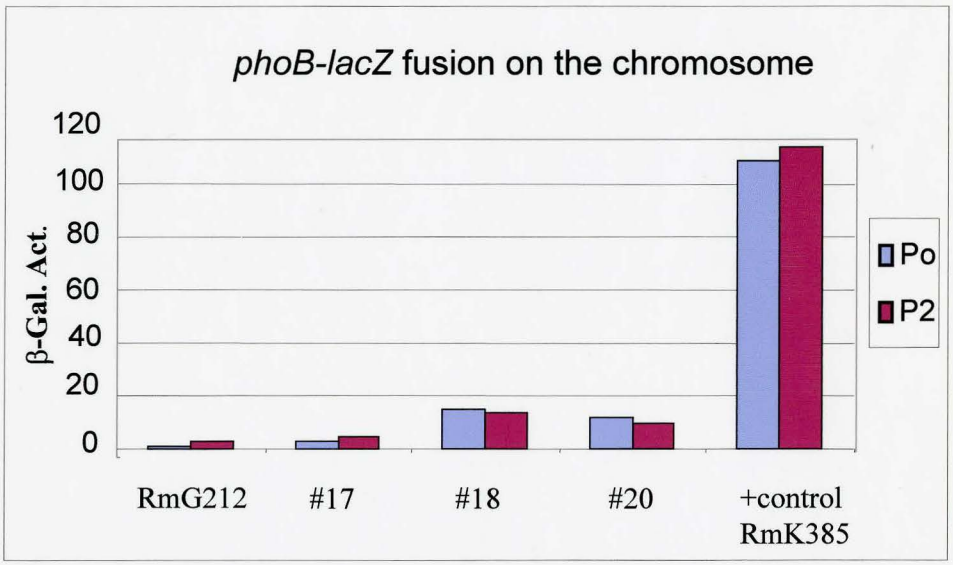


Figure 8. PhoB expression in MOPS minimal medium without phosphate sources (P_0) and 2mM phosphate sources (P_2). β-galactosidase activity and alkaline phosphatase activity were measured after cells growed 16 hours in the specific medium. Tn5-B20 insertions were marker exchanged into RmG212 (wildtype and LacZ-) . #20 and #18 plasmids, *LacZ* with the same direction of *phoB* gene. #17 plasmid, lacZ with opposite direction of *phoB*. Positive control is *S. meliloti* strains RmK385 which can continuously express *lacZ* in both P_2 and P_0.

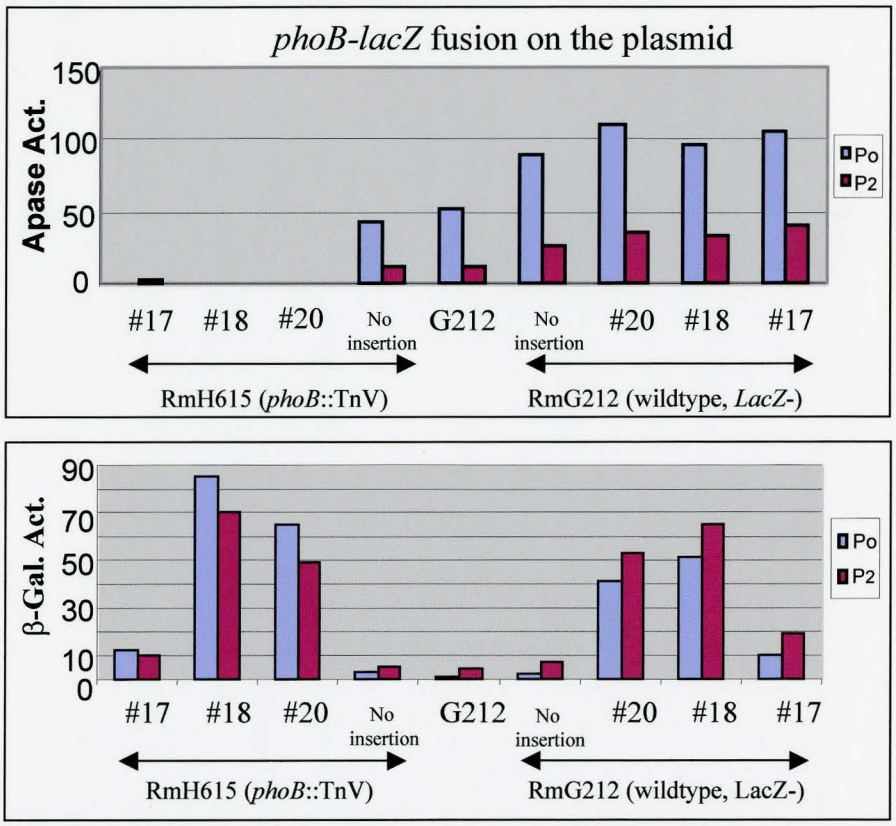


Figure 9. β-galactosidase activity and alkaline phosphatase activity were measured. Plasmid pTH284 plasmid carrying Tn5-B20 insertions were transferred into RmG212 (wildtype and *LacZ*-) and RmH615 (*phoB*::TnV mutant). #20 and #18 plasmids, *LacZ* with the same direction of *phoB* gene. #17 plasmid, l*acZ* with opposite direction of *phoB*. Wildtype without lacZ fusion indicated as "no insertion". RmG212 was wildtype *S. meliloti* strain. MOPS minimal without phosphate sources indicated as P_0, with 2mM phosphate sources indicated as P_2. Cell were grow for 16 hours before assay.

2. Sequence *pstA-pstB* gene and identify the phenotype of *pstB* mutants and investigate *pstB* regulation.

In *E. coli*, the *pst* system is responsible for phosphate transport as well as phosphate regulation. Any mutation in *pstSCAB-phoU* gene cluster lead to constitutive *phoA* expression. *pstSCAB* expression is regulated by *phoB-phoR* two component system. Prior to this study, we were not aware whether the *pstSCAB* genes were present in *S. meliloti*. Our interest in this project came from the discovery of *pstA-pstB* like gene in *S. meliloti* which origined from the construction and sequencing of plasmid pTH301.

1. Plasmid pTH301 consists of the *Sal*I-*Hind*III region from a phoU::TnV insertion as outlined schematically in Figure 10. This plasmid was constructed by Sylvie Bardin (in this lab). Single strand sequence from pTH301 *Sal*I end was obtained using –48 universal primer:

5'---AGCGG ATAAC AATTT CACAC AGGA—3'

GenBank searches with this sequence revealed the presence of a *pstB*-like gene. Figure10 shows the map of pTH301 and the information concerning *pstB* sequence homology.

2. Construct of pTH628, presence of a *pstA*-like gene (Figure 11). Plasmid pTH284 was isolated from cosmid library of *S. meliloti* on the base of its ability to complement *phoB* mutants strains (by Bardin SD). A restriction map of pTH284 is shown in (Figure 11). The 6.5kb *EcoR*I-*Kpn*I fragment from pTH284 was subcloned into pBBR-5 to give plasmid pTH628. When pTH628 was transferred into the *phoB3*::TnV mutant strain, RmH615, it restored this strain's ability to synthesize alkaline phosphatase (Blue colonies on LB-X-phos). Hence pTH628 complements the *phoB3*

mutation. The 2.8kb *Hind*III-*Eco*RI fragment from pTH628 was subcloned into the suicide plasmid pK18GIImob to give pTH642 (Figure 11). The DNA sequence from the *Eco*RI site of pTH642 was ontained using the –48 universal primer and analysis of this sequence revealed a *pstA*-like gene in the region upstream of *pstB* (Figure 11).

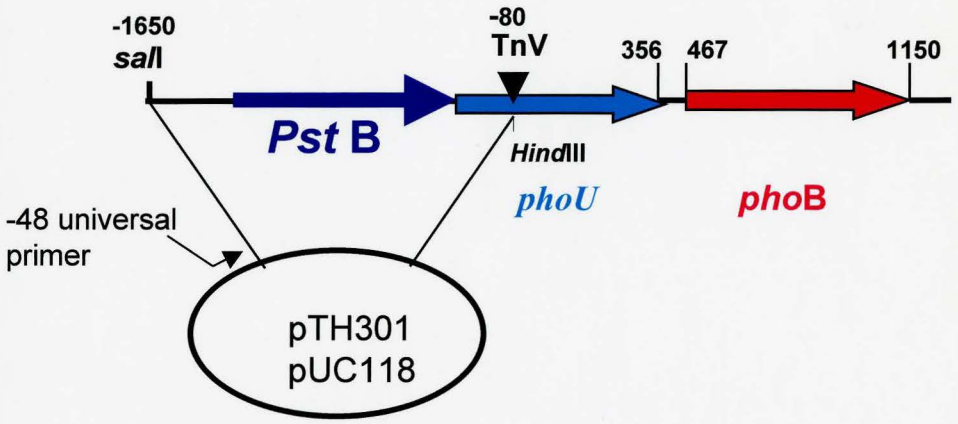
3. In *E. coli*, *pstSCAB-phoU* form one operon which is part of the *pho* regulon and hence regulated by the *phoB/phoR* two component system. *pstSCAB* encode a high affinity ABC type phosphate transport system. *PstSCAB* mutation led to constitutive alkaline phosphatase expression (Wanner BL, 1996). To identify possible functions of the *S. meliloti pstAB*-like genes with respect to symbiotic N2-fixation and phosphate transport and regulation, I disrupted the *pstAB*-like gene using *lacZ-Gm* cassette as outlined in Figure 11. This cassette allows to generate transcriptional fusions to *lacZ* and it is important to note that the *aacC*I promoter is very strong and transcribes genes downstream from the insertion site (Becker 1998 and personal communication). The *lacZ-Gm* cassette was inserted into *pstB* in the *Sma*I site in both orientations. When inserted in the same transcriptional direction with the interrupted gene, the *aacC*I promoter transcribes the downstream genes and make a non-polar mutation. The opposite orientation blocks the transcription of downstream gene and generates polar mutations. This turns out to be a very useful tool for investigating gene expression, especially when the target gene forms an operon together with other genes. When the pTH642-*lacZ*•*aacC*I ligation products were transformed into *E. coli DH5α* and select on LB with X-Gal_{40}, Gm_{10}, Km_{25}, the bule colonies carried the same direction insertion and the white colonies were in the opposite direction insertion. The plasmids carrying the two directions insertions were

double checked by restriction analysis. These plasmids were transferred into the *lacZ⁻ S meliloti* strains RmG212 and transconjugants were selected on LB Gm_{40}, Sm_{400} plates. Single cross-over recombinants were distinguished from double cross-over recombinants by screening for the Km/Nm suicide plasmid marker. Only 1-2% of the recombinants were Nm sensitive. We also attempted to screen for double cross-over recombinants through loss of the GusA suicide plasmid marker. this was done by checking for the recombinants on LB plate with GusA substrate, X-Gluc 50ug/ml. After 3-4 days growth, put the plates in 4°C for 3-4 days, check the clones color (white clones for double cross-over and blue clones for single cross-over). It turned out that all the recombinations are white clones on the X-Gluc50 plate.

4.. I also interrupted the *pstB* gene by inserting the *ΩSp* fragment into pTH642 in the *SmaI* site. This gave plasmid pTH665. I transferred pTH665 into the *lacZ⁻ S meliloti* strains RmG212 and transconjugants were screened for double cross-over recombination in the same way as outlined for lacZ-Gm cassette insertion.

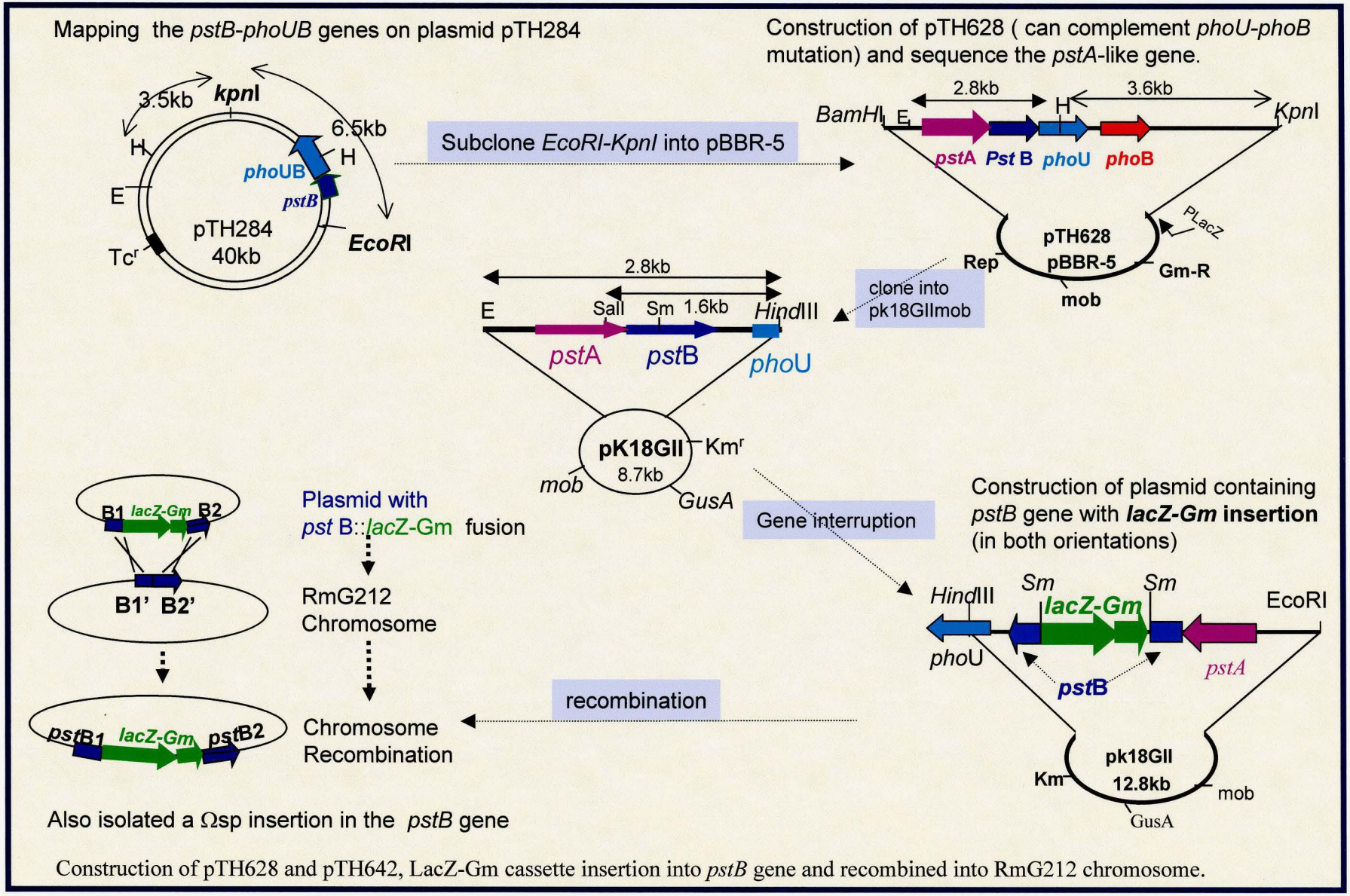
5.. To further analyse whether the above constructs had recombined at the appopriate location in the S. meliloti chromosome, I measured the ϕM12 mediated co-transduction frequency of *LacZ-Gm* cassette and TnV transposon which was inserted in the *phoB* gene of *S. meliloti* (RmH615). Considering that the distance of TnV insertion in *phoB* and the *LacZ-Gm* cassette insertion in *pstB* is only about 1.5kb, the cotransduction frequency was expected to be very high (above 95%, Figure 5). I made a ϕM12 RmH615 lysate. Then transduced into RmK385 (*pstB::LacZ-Gm*, Same direction), RmK386 (*pstB::LacZ-Gm*, opposite direction) and RmK391 (*pstB:: Ωsp)* respectively (select on LB Nm_{200}). Then 120 transducants were patched on Nm200 and Gm40 (for *LacZ-Gm*) or Nm200 and Sp_{50}. None of them were Gm_{40} resistant or

Figure 10. **Identify the *pst*B-like gene of *S. meliloti*.**



BlastX GenBank search with the *pstB*-like sequence:

1. phosphate transport protein PstB - *Escherichia coli*, Identities = 68/137 (49%),
2. Putative phosphate specific transport complex component (*pstB*) [*Methanococcus jannaschii*] Identities = 84/131 (64%)
3. phosphate-specific transport component *pstB* *Pseudomonas aeruginosa* Identities = 81/145 (55%)



Construction of pTH628 and pTH642, LacZ-Gm cassette insertion into *pstB* gene and recombined into RmG212 chromosome.

Sp_{50} resistant. This indicated that the recombination was happened at the right place of *pstB* gene on the chromosome.

6. To investigate if *pstB* expression is regulated by phosphate concentration and what is the phenotype of a *pstB* mutant? In free-living cells, alkaline phosphatase activity and β-galactosidase activity of *pstB-lacZ* fusion mutants was measured in MOPS minial medium without phosphate sources (P_0) and 2mM of phosphate source (P_2). All the glass were used in this experiment were rinsed by 6M HNO_3 to get rid of the residual phosphate. The methods were given in the chapter II. Figure 12 give the results for these analyses.

In phosphate limiting conditions (P_0), when *lacZ-Gm* was inserted in the same direction as *pstB,* the recombinant was Apase+, when the cassette was inserted in the opposite direction to *pstB*, the recombinant was Apase-. In phosphate excess, almost no Apase activity was detected in both insertion directions. Previous studies have shown that *phoU* and *PhoB* mutants were both Apase- and it was presumed that *phoUB* are in one operon. The results in Figure 12 also indicate that *pstB-phoUB* are in one operon. In summary, *pstB* insertions lead to polar effects on the downstream *phoUB* genes. Apase activity in the pstB mutant is dependent on the orientation of the *lacZ*-Gm cassette insertion. Also, interrupted *pstB* by *ΩSp* insertion led to polar effects on *phoUB,* as shown by the Apase minus phenotype.

When the *lacZ*-Gm cassette was inserted in the same direction as *pstB*, the β-galactosidase activity of *PstB-lacZ* fusion mutants were the same in both P_0 and P_2 conditions. *pstB-lacZ* insertion in opposite direction generate almost no LacZ activity. We concluded that *pstB* expression is not regulated by phosphate concentration. But is *pstB* expression was regulated by PhoB? In the next section of this work, we will

address this question.

Conclusions:

1. *pstB* expression is not regulated by phosphate concentration.
2. Interrupted *pstB* by *Ωsp* insertion lead to Apase minus phenotype.
3. Apase activity in the *pstB* mutant is dependent on the orientation of the *lacZ-Gm* cassette insertion.
4. *pstB-phoUB* are in one operon.

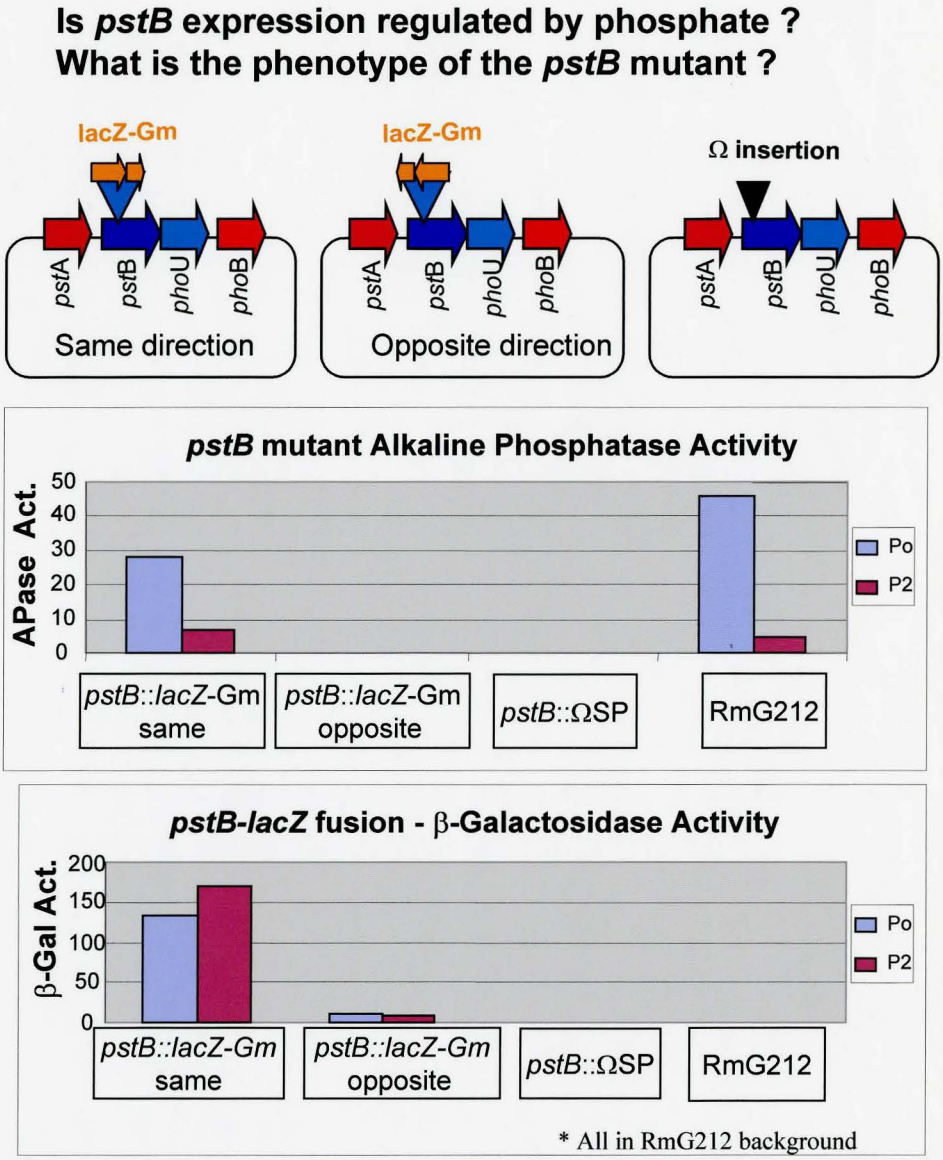


Figure 12. *pstB* gene expression were measured by β-galactosidase assay. The phenotype of *pstB* mutants were determined by Alkaline phosphatase in the free-living cells in MOPS minimal medium without phosphate sources indicated as P_0, with 2mM phosphate sources indicated as P_2. Cell were grow for 16 hours before assay. With RmG212 as control (wildtype and *LacZ-).* The proper genotype construction are shown as above.

3. *pstB* mutation can suppress *phoCDET* mutation into Fix+ phenotype. *PstB* mutant has normal Fix+ phenotype.

Previous research has shown that the *phoCDET* genes are required for symbiotic nitrogen fixation. *phoB* is required for *phoCDET* expression but represses *orfA-pit* expression. When inoculated on the host plant, alfalfa, *phoB* mutants formed normal N_2-fixing nodules on alfalfa plants (i.e. Fix+ phenotype). In addition, *phoB* mutations suppressed *the Fix- phenotype of phoCDE*T mutants by increasing expression of *orfA-pit* system. Here we want to address the following questions:

1). What is the symbiotic phenotype of a *pstB* mutant?

2). Can *pstB* mutations suppress *the Fix- phenotype of phoCDET* mutants?

First, *pstB* and *phoCDET* double mutants were constructed. This was done by transducing the *pstB::Ω Sp* insertion from RmK391 into the *phoC*::Tn5 mutant (RmF921) and selected on LB Sp_{50} to give strain RmK399. Alfalfa seedings were inoculated with *pstB* mutants, *pstB-phoCDET* double mutants, *phoCDET* mutants, *phoB-phoCDET* double mutants and wildtype strains (*Rm1021*). Unoculated alfalfa was also included as a negative control. 30 days after inoculation, the symbiotic phenotype of the strains was determined by measuring root nodule acetylene reduction activity and also by plant dry weight determination. Pictures of representative plants are shown in Figure 13. The plant dry weight and acetylene reduction results were shown in Figure 14. *PhoB-phoCDET*, *pstB-phoCDET* and *pstB* mutants inoculated plants grew as well as the wildtype, they were all Fix+. The *phoCDET* mutant was Fix-. The dry weight of plants inoculated with *phoB-phoCDET*, *pstB-phoCDET* and *pstB* mutants almost the same as the dry weight of plant inoculated with the wildtype. Acetylene reduction activity also show the roots from the

plants inoculated with *phoB-phoCDET, pstB-phoCDET* and *pstB* mutants almost were the same acetylene reduction activity as the roots from plants inoculated with wildtype strains Rm1021. The *pstB* mutant, like *phoB* mutant (date not shown here), was Fix+. Clearly, the *pstB* mutation, like the *phoB* mutation, can suppress the Fix$^-$ phenotype of a *phoCDET* mutant.

Conclusions:

1. *pstB* mutants, like *phoB* mutant, formed Fix+ nodules on alfalfa plants.
2. *pstB* mutation suppressed the Fix - phenotype of *phoC* mutant.

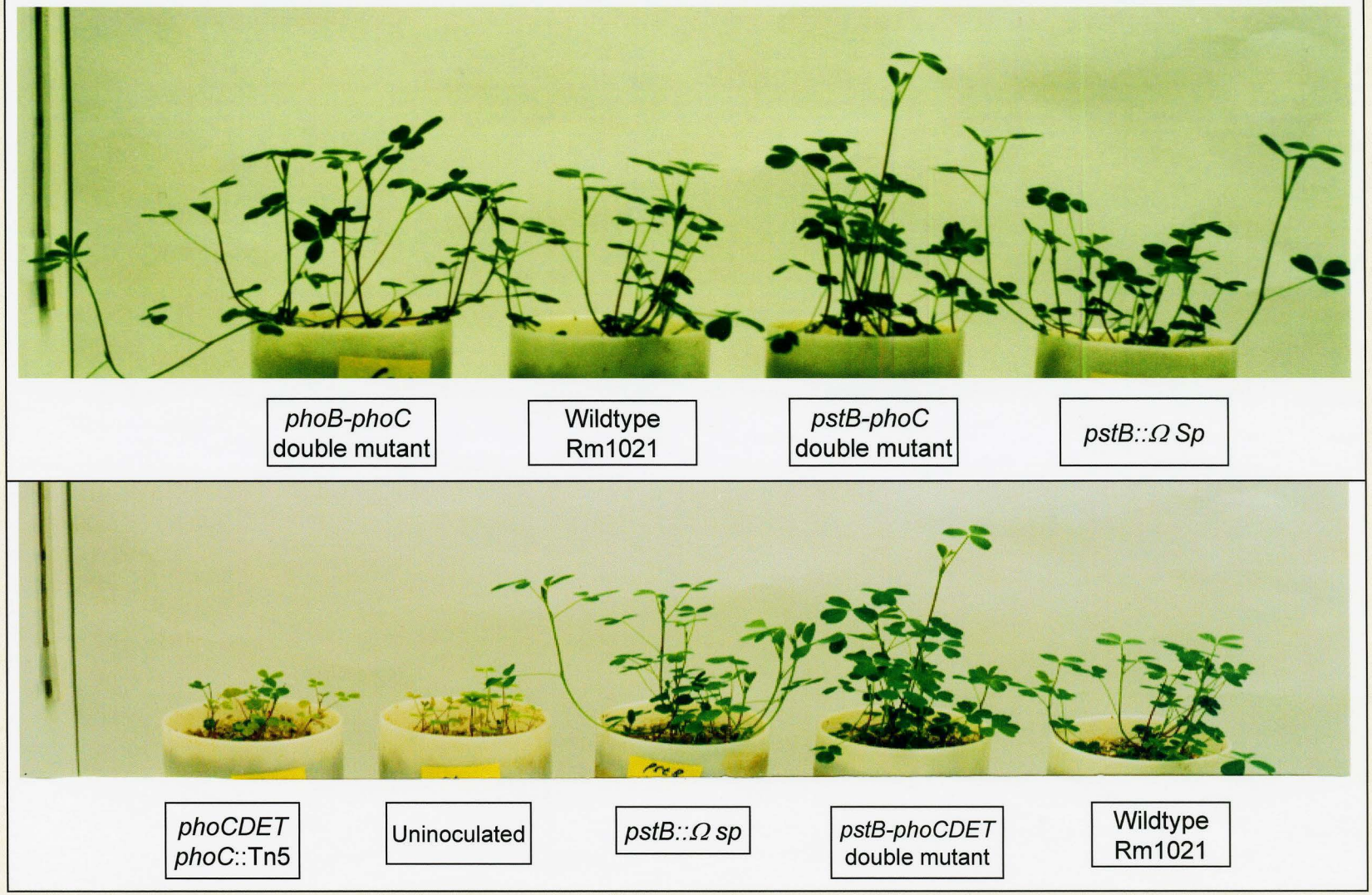


Figure 13. *pstB* and *pstB-phoCDET* double mutants plant test (30 days plant growth).

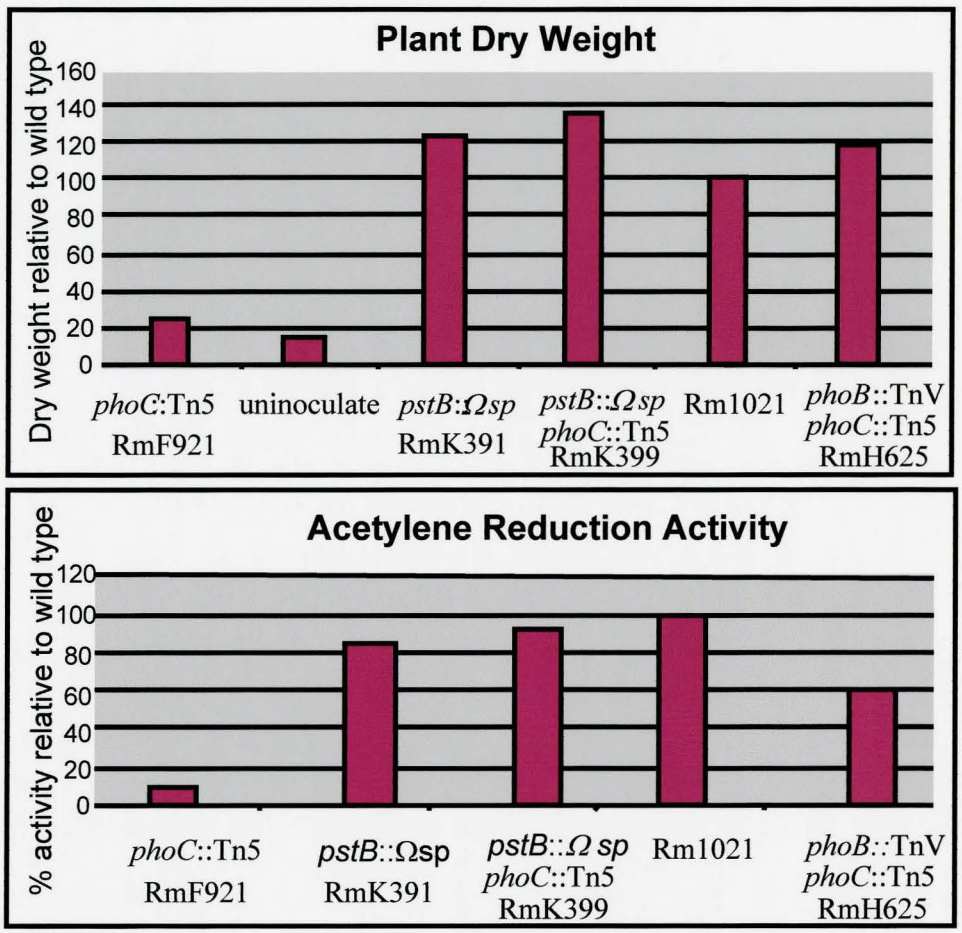


Figure 14. *pstB* mutants and *pstB-phoCDET* double mutants plant test. The plant growed for 30 days after inoculated the indicated strains. The dry weight were measured after plant were dry 3 weeks in the oven until they could not dry any more. The plant root with nodules were used for acetylene reduction assay.

4. In *S. meliloti*, *pstA-pstB-phoU-phoB* is in one operon, *pstB* expression is independent on *phoB*.

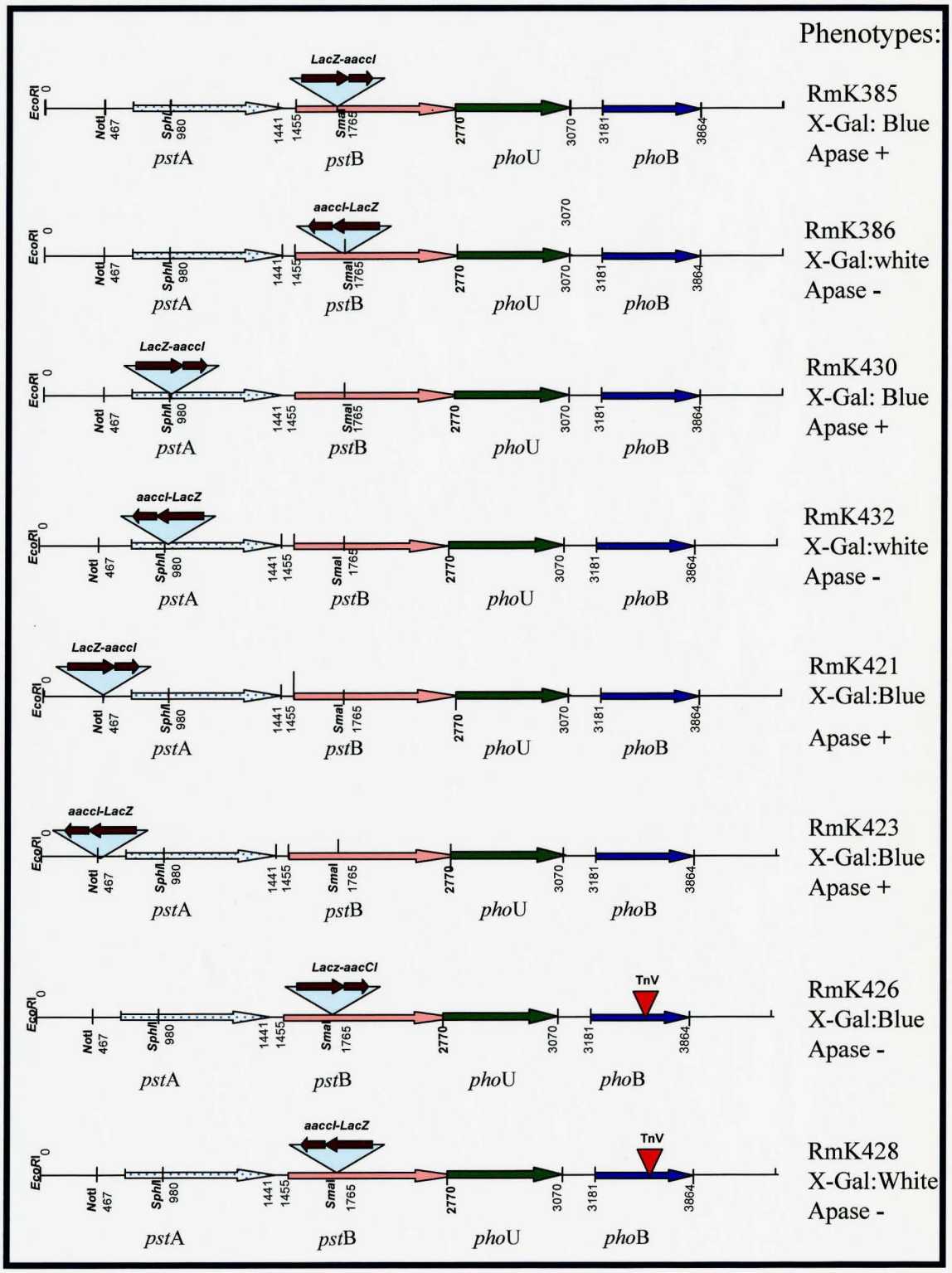
In *E coli, phoB-phoR* are form one operon and function as a two component system to regulate phosphate transport and regulation. *pstSCAB-phoU* form another operon and encoding a high affinity ABC type phosphate transport system. Also the *pst* system is believed to be involved in phosphate regulation. In *S. meliloti*, we presume that *pstA-pstB-phoU-phoB* is in one operon, this is based on the following: 1). pTH628 (Figure 11) complemented *phoUB* mutations, and in pTH628, the *lacZ* promoter of the pBBR-5 vector is on the opposite direction to the direction of transcription of phoUB, which indicates that the 6.5kb DNA fragment includes the promoter for phoUB. 2). *phoU* mutations and a *pstB* mutation have the same phenotype Apase$^-$. 3). a *pstB* mutation, like a *phoB* mutation, can suppress the Fix$^-$ *phoCDET* phenotype. 4). The *pstA* and *pstB* gene open reading frames are very close (14bp) and the *pstB* and the *phoU* gene open reading frame are overlapped. 5), These data promoted us to identify the phenotype of a *pstA* mutant and to determine whether a *pstA* mutation has any affect on *pstB-phoUB* transcription? To generate a pstA mutant, the *lacZ*-Gm cassette was inserted in both orientations into *pstA (SphI* site) and upstream of *pstA* gene (*NotI* site). All the constructs were recombined into *RmG212* chromosome (wildtype, *lacZ*-) and checked the loss of vector DNA by plating on LB medium with X-Glu (GusA substrate) and Nm_{200}. Then these constructs were plated on LB medium with X-$Phos_{40}$ or X-Gal_{40}. The results of this experiment and the corresponding genotype and phenotypes of the various strains are shown in Figure 15.

1). The *LacZ*-Gm cassette inserted inside the *pstA* gene (*Sph*I site) generated the same phenotype as when it was inserted into *pstB* gene (*Sma*I site). Also, when the *lacZ* insertion was in the same direction as the *pstA* gene, lacZ activity were detected on LB plates with X-Gal40 (bule clones). Otherwise, *lacZ* activity could not detected. This result indicates that the insertion in the *pstA* gene is polar on *phoUB* gene.

2). Strains carrying the l*acZ*-Gm insertion in both orientations upstream of *pstA* (*Not*I site) were Apase+. This suggests that the *Not*I site is outside the *pstAB-phoUB* gene operon.

3). In further analysis of the *pstAB-phoUB* gene sequence, we could not find the *pho* box motif. To investigate whether *pstB* expression was regulated by *phoB*? We constructed the *pstB* and *phoB* double mutants by transducing the *phoB*::TnV into the *pstB-LacZ-Gm* fusion of both directions. Appropriate double mutants were identified following a screen of Nm_{200}^{R} transductants. Then the expression of *pstB-lacZ* fusions were measured in both *phoB* wildtype and *phoB*::TnV mutant background (blue or white on LB plates with $X\text{-}Gal_{40}$ and $X\text{-}Phos_{40}$). As shown in Figure 15, *pstB-lacZ* expressed in *phoB-pstB* double mutant when *lacZ* was fused in the same direction as *pstB*. When lacZ was fused in the opposite direction, *pstB-lacZ* expression not detected. This indicated that *pstB* expression is independent of *phoB*.

(Figure 15) Insert *LacZ-Gm* cassette at different site of *pstA-pstB-phoUB* operon lead to different phenotype of alkaline phosphatase



5. Clone *phoR-pstSCAB* gene cluster and identify *phoR* function?

In *E coli*, *phoB/phoR* are key components involved in regulating genes of the *pho* regulon. We were therefore interested in knowing whether *S meliloti* contained a *phoR* like gene and if so, does it play a role in regulating genes involved in phosphate assimilation? Analysis of the *S. meliloti* genome sequence obtained from the international genome project (unpublished) and revealed the *presence of phoR-pstS-pstC*-like genes right upstream of *pstA-pstB-phoU-phoB*. Using an R-prime approach (Figure 16), we cloned a 7.5 kb *Hind*III fragment from *S. meliloti* which included the *phoR-pstSCAB* genes and partial *phoU* gene.

Construction of R-prime plasmid was based on the ability of plasmid $R_{68.45}$ to mobilize the DNA of the host strain at high frequency (Riess 1980). The $R_{68.45}$ derivative pJB3JI was transfered into RmH615 (*phoB*::TnV) and selected on Nm_{200} and Tc_{10}. The resulting strain was used as donor in conjugational mating with a Rif^R *E. coli* recipient MT620. Transconjugants were selected on LB with $Rifampicin_{20}$ and Km_{20} (overnight growth) for isolation of plasmids containing the transposon TnV (Km/Nm). Five R-prime plasmids were isolated in this way. Referring to the TnV restriction map and the upstream gene sequence and restriction sites in *phoR-pstS-pstC* region (Appendix II), I subcloned the 7.5kb *Hind*III gene fragment from the R-prime plasmid #7 into pUC119 vector. Further restriction analysis of the 7.5kb gene fragment proved that it was the fragment containing *phoR-pstSCAB* and partial *phoU*. To double check that the fragment I cloned was indeed the *phoR-phoB* gene region, I sequenced the fragment from the *phoU* gene end using the -48 universal primer. The sequence confirmed that the fragment I cloned from the #7 R-prime plasmid exactly contained the *phoR-pstSCAB* and partial *phoU* (pTH691, Figure 17).

Figure 16. **Strategy to clone *phoR-pstSCAB-phoU* gene fragment from *S. meliloti***

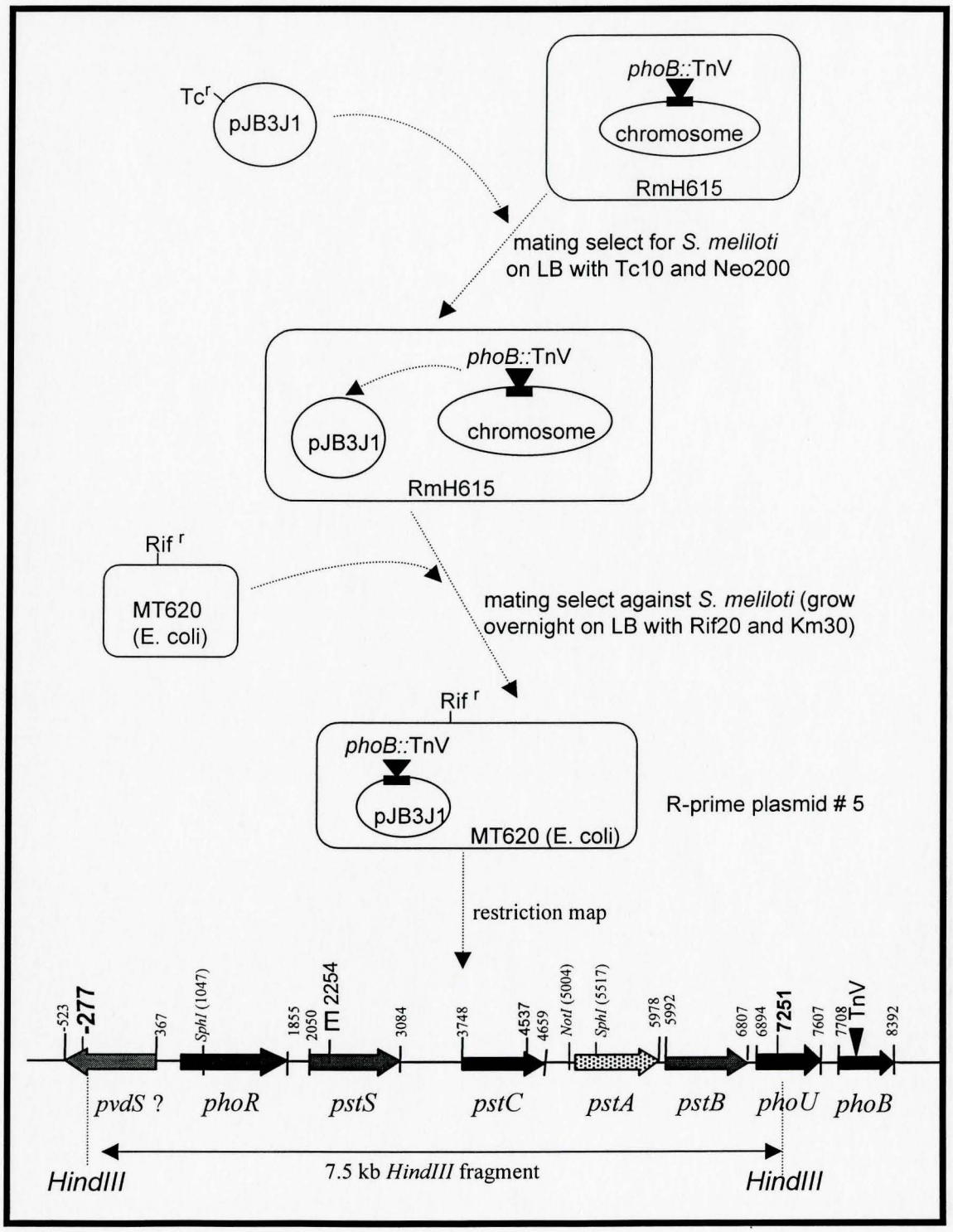
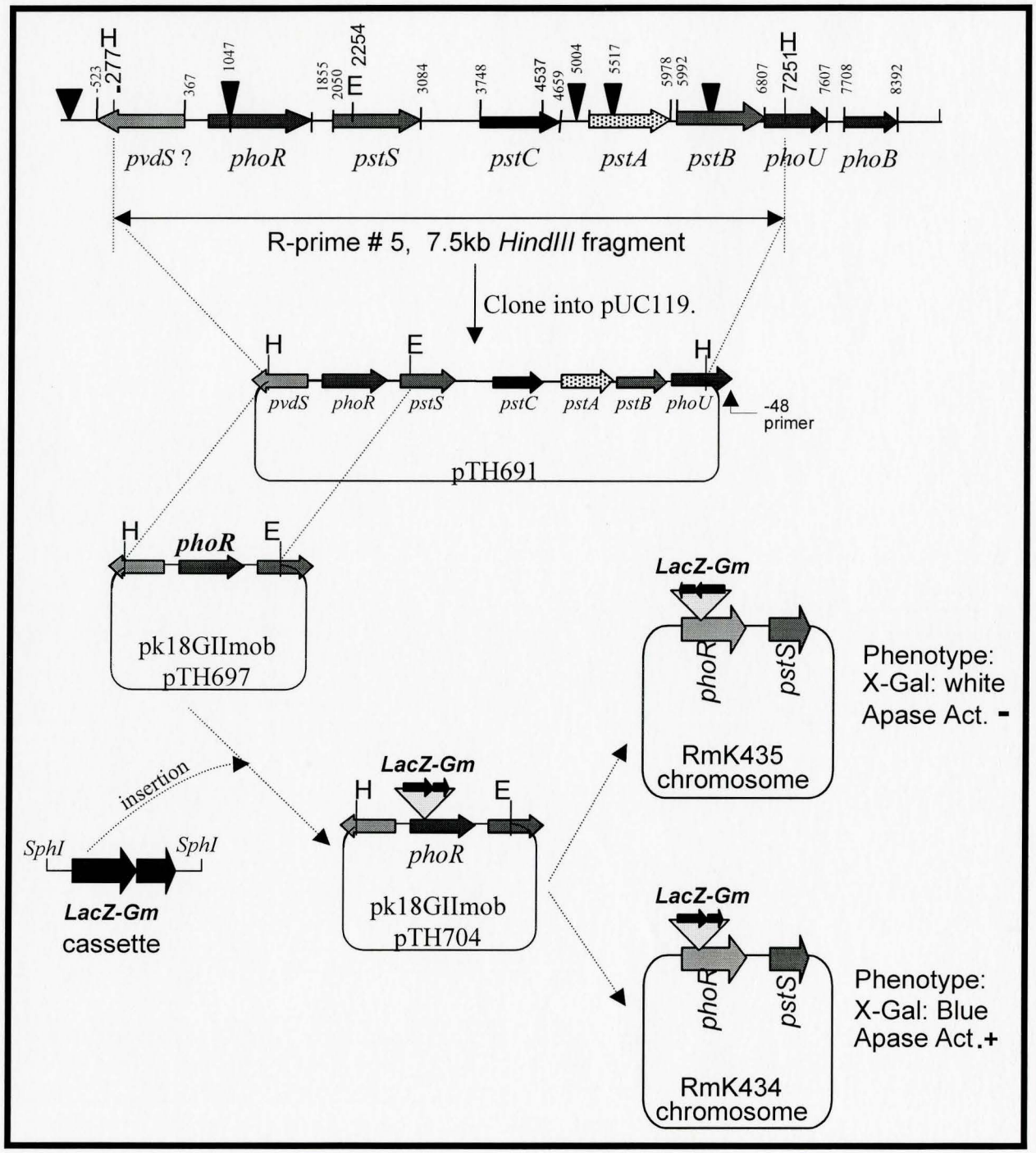


Figure 17.

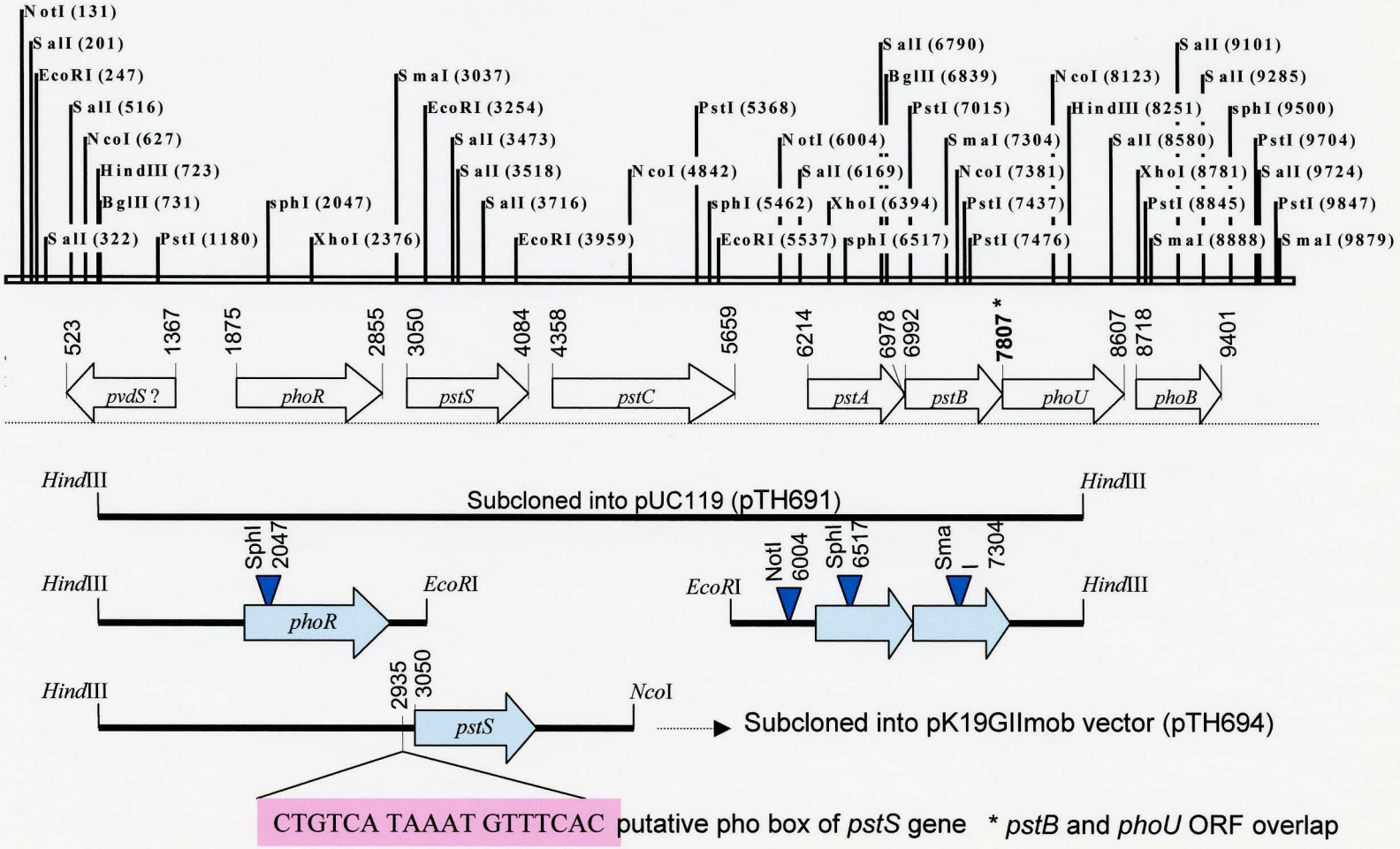
Strategy for constructing *S. meliloti phoR-LacZ-Gm* gene fusions



To make the *phoR* mutation/*lacZ-aaccI* fusion, the 2.5 kb *HindIII-EcoRI* fragment which included the *phoR* gene was subcloned from pTH691 into the pk19GIImob suicide vector. The *lacZ-Gm* cassette was then inserted in both orientations into the *SphI* site of *phoR*. These insertions were recombined into the *RmG212* chromosome in the same way as described for the *pstB* gene. The *phoR* mutant (RmK434, *lacZ-Gm* insertion in the same directon of *phoR*) formed blue colones on LB plate with X-Gal_{40}, and it was Apase+ (blue clones) on LB plate with X-$Phos_{40}$. The *phoR* mutant (RmK435, *LacZ-Gm* insertion in opposite direction of *phoR*) formed white colones on LB with X-Gal_{40}, and was Apase- (white clones).

In *E. coli*, most *phoR* mutations are Apase- (Wanner BL, 1980). PhoB activation is subject to cross-talk, hence without *phoR*, PhoB can be activated by CreC and Acetyl phosphate (Figure 3). In *S. meliloti*, the role of *phoR* appears complex. In this study, the phenotype of PhoR insertion mutants was dependent on the orientation of *lacZ-Gm* cassette. Insertion in same direction as *phoR* were Apase+, while insertion in opposite direction as *phoR*, were Apase-. We note that *pstS* and *pstC* genes are located downstream of *phoR*. It is possible that the *phoR* mutation influences *pstS* and *pstC* expression. Further, the powerful *aaccI* promoter could drive *pstS* expression? Further experiments are needed to define *phoR* function and its regulation. For this purpose, pTH693 which contained the *pstS* gene fragment were constructed by deleting the two *NcoI* fragment from pTH694 (Figure18). Further analysis of *pstS-pstC* function and regulation would help to understand this complicated process.

Figure 18. Restriction map of *phoR-pstSCAB-phoUB* genes region, genes open reading frame(ORF) & interruption



6. *pho* box analysis of the *phoR-pstSCAB-phoU-phoB* region.

In *E. coli*, many genes involved in phosphate transport and assimilation are part of a regulon referred to as the *pho* regulon. Expression of these genes is subject to postive regulation by the transcriptional activator PhoB, PhoB binds to a site in the –40 region of the promoter referred to as the *pho* box. In *S meliloti*, an equivalent *pho* box was found in the promoter of the *phoC* gene (2 copies) and *orfA-pit* genes (Bardin et al, 1996, 1998). Another putative *pho* box was found in the promoter region of *orfA-pta-ackA-fabI* (Summers 1998) and *phnG* gene (McLean PA, unplished, NCBI gene bank M96263). We wanted to know if there were any *pho* Boxs in the *phoR-pstSCAB-phoU-phoB* region. The putative *pho* boxs identified in *S. meliloti* were listed as follows:

S. m--phoC1. CTG TTA CAGAAC CTACAC

S. m--phoC2. CTG ACA CTGCGC TTTCAT

S. m--orfA-pit. CTG TCA TAAAAC TGTCAT

S. m--phnG. ATG TCA CAAGCC TGTCAT

S. m—orfA-pta TTG TCA AACCGC CGTAAC

***S. meliloti pho* box consensus: CTG TCA TAAAT CTG TCAT**

E.coli pho box consensus: CTG TCATA A(T) A T(A) CTGTCA C(T).

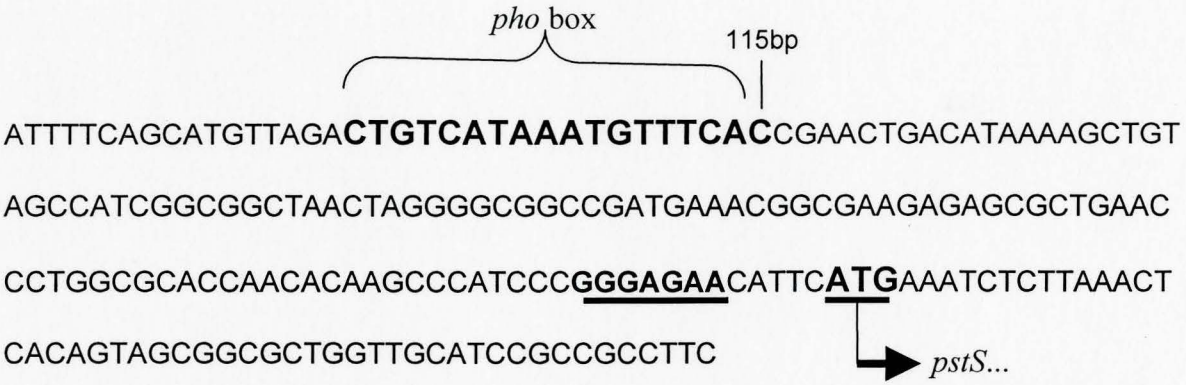
Refered to all the *pho* box motif identified in *S. meliloti* and *E. coli*, Dr Richard Morton in this department developed the *pho* box motif search program. He collected all the available *pho* boxes identified in *S. meliloti* and also the *E. coli pho* Boxes motif (18 base pairs sequence), then generated a Matrix with the frequence of the four nucleotides at each position of the *pho* box. Employing this matrix the program searches nucleotide sequences for 18 bp region with high similarity or scores relative

to the matrix. Using this program, I searched the *phoR-pstSCAB-phoU-phoB* region and found that there is no obvious *pho* box in the *pstA-pstB-phoU-phoB* operon. This makes sense because our data shown that expression of this operon is not regulated by the *phoB* gene or varying phosphate concentrations. A very clear *pho* box was found located at 115bp upstream of translation start site of the *pstS* open reading frame (downstream of the *phoR* gene). Figure 19 and 20 show the *S. meliloti pho* box motif and the putative *pho* box of *pstS*. The functional significance of this site remains to be established. This could be persued through the generation of a *pstS-lacZ* gene fusion and the analysis of this fusion in *phoB* wildtype and a *phoB* mutant background.

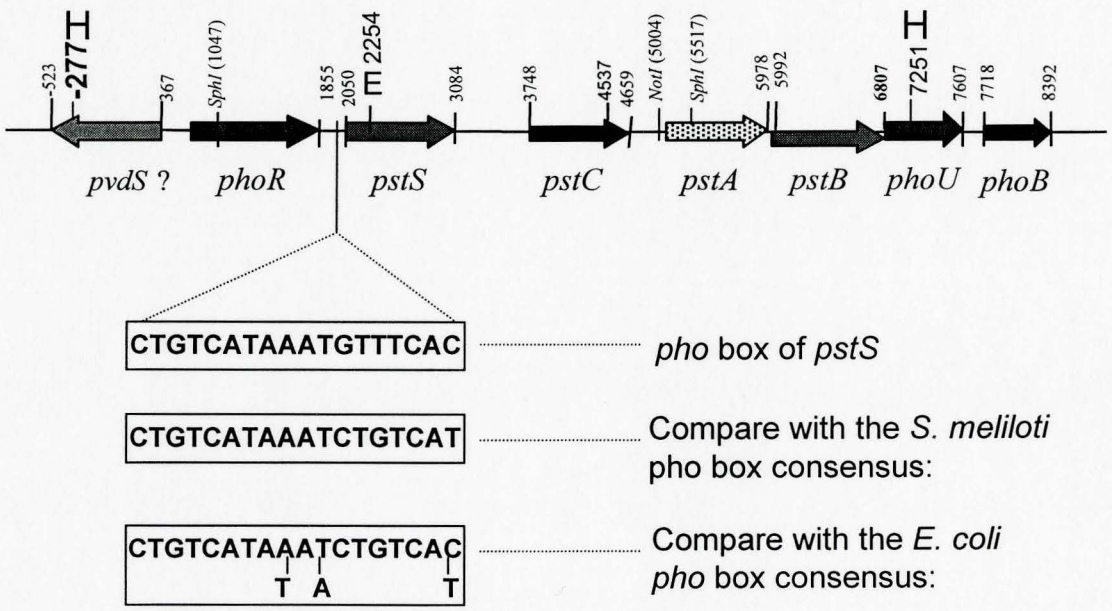
pho box motif search																		
gene name	pos #1	pos #2	pos #3	pos #4	pos #5	pos #6	pos #7	pos #8	pos #9	pos #10	pos #11	pos #12	pos #13	pos #14	pos #15	pos #16	pos #17	pos #18
Rm:orfA-pta	T	T	G	T	C	A	A	A	C	C	G	C	C	G	T	A	A	C
Rm:phnG	A	T	G	T	C	A	C	A	A	G	C	C	T	G	T	C	A	T
Rm:phoC1	C	T	G	A	C	A	C	T	G	C	G	C	T	T	T	C	A	T
Rm:phoC2	C	T	G	T	T	A	C	A	G	A	A	C	C	T	A	C	A	C
Rm:orfA-pit	C	T	G	T	G	G	G	A	A	A	G	C	C	G	T	T	T	T
Ec:phoA	C	T	G	T	C	A	T	C	A	C	T	C	T	G	T	C	A	T
Ec:phoB	C	T	G	T	C	A	T	A	A	A	G	T	T	G	T	C	A	C
Ec:phoE	C	T	G	T	A	A	T	A	T	A	T	C	T	T	T	A	A	C
Ec:phoH	C	T	G	T	C	A	T	C	A	C	T	C	T	G	T	C	A	T
Ec:phnC	C	T	G	T	T	A	G	T	C	A	C	T	T	T	T	A	A	T
Ec:pstS1	C	T	G	T	C	A	T	A	A	A	A	C	T	G	T	C	A	T
Ec:pstS2	C	T	T	A	C	A	T	A	T	A	A	C	T	G	T	C	A	C
Ec:ugpB1	T	T	G	T	C	A	T	C	T	T	T	C	T	G	A	C	A	C
Ec:ugpB2	C	T	A	T	C	T	T	A	C	A	A	A	T	G	T	A	A	C
Ec:ugpB3	A	A	G	T	T	A	T	T	T	T	T	C	T	G	T	A	A	T
Frequencies (excludinh gaps) adjusted by adding 0.5 for zero count																		
A	0.13	0.06	0.07	0.13	0.07	0.84	0.07	0.58	0.4	0.53	0.27	0.07	0.03	0.03	0.13	0.32	0.88	0.03
T	0.13	0.88	0.07	0.81	0.2	0.07	0.6	0.19	0.27	0.13	0.33	0.13	0.75	0.25	0.81	0.07	0.06	0.5
C	0.71	0.03	0.07	0.03	0.67	0.03	0.2	0.19	0.2	0.27	0.13	0.77	0.19	0.03	0.03	0.58	0.03	0.44
G	0.03	0.03	0.84	0.03	0.07	0.07	0.13	0.03	0.13	0.07	0.27	0.03	0.03	0.69	0.03	0.03	0.03	0.03
cinsensus sequence	C	T	G	T	C	A	T	A	A	A	T	C	T	G	T	C	A	T
consensus score = 1.23.																		

Figure 19. S. meliloti pho box motif.

Figure 20. *pho* Box search of the *phoR-pstSCAB-phoU-phoB* region.



The *pstS* translational start site indicated by **ATG,** also the Shine-Dolagro sequence was indicated by blue color. The *pho* box was located at 115bp upstream of *pstS* start site.



7. Comparison of *pstSCAB* gene clusters in different bacteria (Figure 21).

The *pstSCAB* genes play a important roles in phosphate uptake and signal transduction in bacteria. Here we survey the distribution of the *pstSCAB* genes in several bacterial genomes.

In *E. coli*, *pstSCAB-phoU* is in one operon. Any mutation in this operon leads to constitutive alkaline phosphatase expression. Phosphate uptake and the regulation of phosphate uptake are controlled by a two component system, *phoB-phoR*, which also forms one operon. This operon is phosphate regulated and autoregulated (Wanner BL, 1993). *pstS* has two *pho* boxes in the promoter region, each *pho* Box is functional independently (Kimura, 1989).

In *Streptococcus pneumoniae*, The Pst system is involved in P assimilation (Novak R, 1999). A *pstB* insertion leads to a decrease in P_i uptake, genetic transformation, and autolysis. Mutagenesis of the *phoU* gene did not lead to this phenotype. The *Pst* system might be involved in a P-independent signaling pathway. Expression of the two-component regulatory system *PnpR-PnpS* (regulate phosphate uptake) was not influenced by different phosphate concentrations and might not linked to the pneumococcal *Pst* system. The Pst system in pneumococcal play a more complex physiology role than in that of *E. coli* (Novak R, 1999).

***Burkholderia* sp.** contains a genomic region similar to the *pst* operon of *E. coli* in sequences as well as in the order and number of genes and hence has the potential to take up Pi from its environment. The *pst* genes probably constitutes an operon (Ruiz-Lozano,1999).

In *Caulobacter crescentus*, The gene cluster of *phoR-pstCAB-phoU-phoB* involved

in the stalk elongation, cell division and phosphate regulation. *pstCAB-phoU-phoB* could be one operon. *PstS* gene was far away from this gene cluster. Both *pstS* and *phoB* mutants were deficient in phosphate transport. *phoB* mutants were unable to elongate stalks during phosphate starvation, whereas *pst* mutants made long stalks in both high- and low-phosphate media. *pstS* transcription is dependent on *phoB* (Gonin et al, 2000).

Bacillus subtilis The *pst* system is transcribed from a single promoter and encodes high-affinity Pi uptake system (Takemaru et al, 1996). Expression from this promoter was induced >5,000-fold upon phosphate starvation and regulated by the *PhoP-PhoR* two-component regulatory system. The *pst* operon is not involved in Pi regulation. The *phoU* gene is missing, while there are two copies of *pstB* genes. *PhoPR* operon shows low constitutive *phoPR* transcription in a *phoP* or *phoR* mutant strain (Qi et al, 1997).

In Pseudomonas aeruginosa, the gene order of the phosphate-specific transport (*pst*) operon is *pstC-pstA-pstB*, and a well-conserved *pho* box sequence (16/18 bases identical) exists in the promoter region, and *pstS* gene locates far from this operon (Nikata et al, 1996. genBank accession AB017492). Expression of the *pst* operon at high levels did not increase P(i) uptake in *P. aeruginosa*. *phoB* has a very well conserved *pho* box in the promoter of the gene. The sequence data also predicted the presence of *phoR* downstream of *phoB* (Anba J.1990, Filloux A, 1988).

Enterobacter cloacae The pst system gene order were same as in *E. coli, pstSCAB-phoU*. Also there is a *pho* box before the start site of *pstS* genes. *E. cloacae Pst* system is required for Pi chemoreception. *phoBR* gene was not yet identified so far (Kusaka et al, 1997).

In *M. tuberculosis*, the situation was more complicated. 1). *pstB, pstS-1, pstC-1, pstA2* form one potential operon encoding a putative phosphate transporter Pst system. 2). *pstS-3, pstC-2* and *pstA1* are included in a cluster encoding a second putative ABC phosphate transport system. 3). *PstS2* and *MbK* gene are in the third gene cluster. The three *M. tuberculosis* proteins *PstS-1, PstS-2*, and *PstS*-3, share a similar function and were all induced under phosphate starvation. These *pstS* gene duplications may be a subtle adaptation of intracellular pathogens to phosphate starvation in their alternating growth environments (Lefevre P, 1997). A putative two-component system, *mtrA-mtrB*, was also isolated. The *mtrA* gene product displayed high similarity with typical response regulators, including *AfsQ1, PhoB, PhoP,* and *OmpR*. The *mtrB* gene product displayed similarities with the histidine protein kinases *AfsQ2, PhoR*, and *EnvZ* and other members of this class of proteins (Via LE, 1996). Putative *pho* box were not identified in this organism.

V. cholerae responds to phosphate limitation in a way similar to *E. coli*. Phosphate transport system was not investigated yet. *pho* regulon has a role in adaptation of *V. cholerae* to the intestinal environment. The *phoBR* regulatory region contains a putative consensus *pho* box located at region -35 of the promoter, suggesting that *phoBR* is under control of PhoB and, similar to *E. coli*, is phosphate regulated. The *V. cholerae pho* regulon *in vivo* may not be regulated by inorganic phosphate levels alone (von Krüger, 1999).

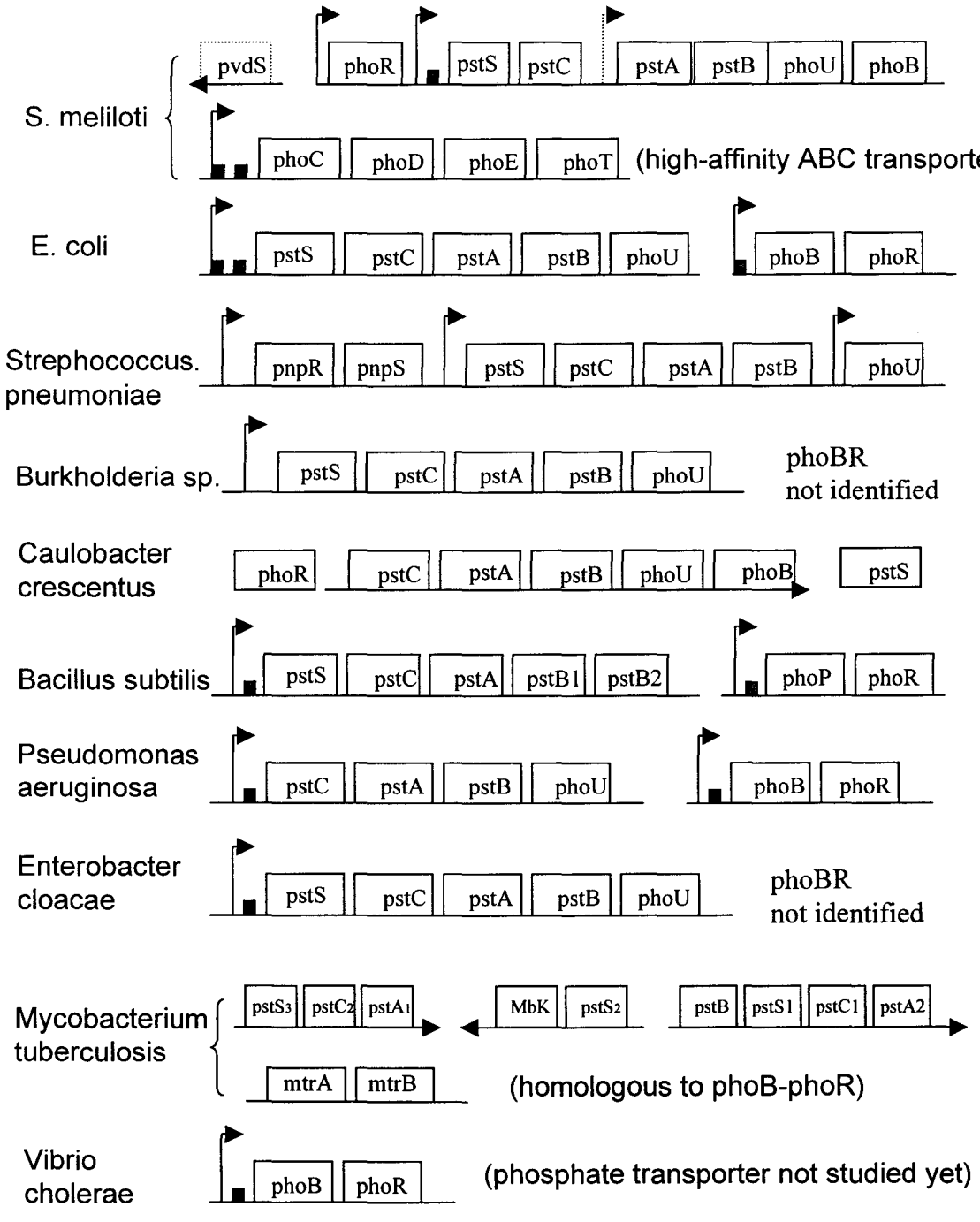


Figure 21. comparison of pst gene cluster in different bacteria. ■ Indicate *pho box*. ▶ Indicate the promoter and its direction. → Indicate the transcriptional direction of the gene cluster.

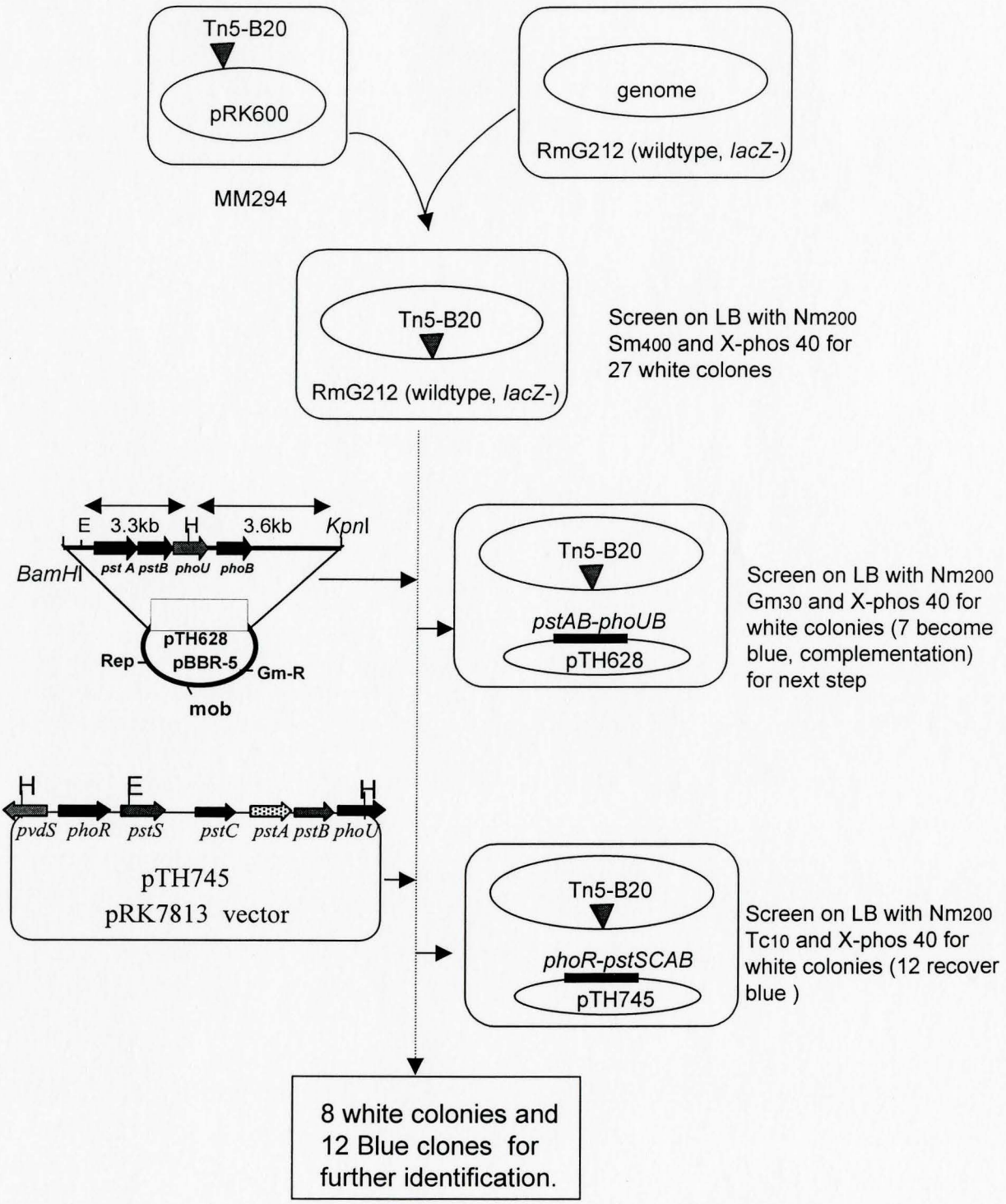
8. Identify other phosphate regulation genes.

In microorganism, it appear likely that there is cross-regulation between genes whose expression alters in response to varying phosphate concentrations and other physiological conditions. PhoB is phosphorylated and activated by PhoR, CreC as well as acetylphosphate (reviewed by Atsuo Nakata, Figure 18). In 1999, Summers et al identified a locus from *S. meliloti*, which include *orfA-pta-acka-fabI*. *pta* gene encodes phosphotransacetylase and *ackA* encodes acetate kinase. These two enzymes are required to synthesize acetyl phosphate, which is a potential signaling metabolite to phosphorylate PhoB. Interestingly, this operon was induced by limited phosphate and is depend on *phoB.* There is a putative *pho* box in the promoter region of this operon. The *ackA* and *pta* mutants form normal Fix^+ nodules. Whether acetyl phosphate can phosphorylate PhoB or other response regulators in *S meliloti* needed to be examined further.

To identify other genes involved in the regulation of phosphate assimilation in *S. meliloti*, a random Tn5-B20 insertion/fusion mutagenesis of *RmG212* was carried out (Figure 22). *E. coli* MM294 (pRK600::Tn5-B20) was transferred into *RmG212* and transconjugants were screened on LB with Nm_{200} and Sm_{400} and X-$phos_{40}$. *RmG212* forms blue colones on LB X-$phos_{40}$. Twenty-seven white colonies were obtained from several matings. Following purification, plasmids pTH628 (*pstAB-phoUB*) was transferred into the 27 mutants. Seven of these transconjugants formed blue colonies on LB X-$phos_{40}$ plates and these likely carry mutations in the *pstAB-phoUB* genes. Plasmid pTH745 carrying *phoR-pstSCAB* genes was transferred into the 20 mutants and 12 of the resulting transconjugants turned blue on LB X-$phos_{40}$ plates. Hence these 12 mutants likely carry mutations in the *phoR-pstSCAB* region.

Further work remains to be done to identify the remaining 8 mutants although at least some are likely located in the *phoA* gene region.

Figure 22. Strategy to identify other phosphate regulation genes



Chapter IV. Discussion and Future Studies

1. Comparision of *E. coli* PstSCAB and *S. meliloti* PhoCDET system:

In *E. coli*, PstSCAB system is ABC type high affinity phosphate transport system. Also *pstSCAB* has a role in phosphate regulation, many mutations in this system lead to constitutive expression of alkaline phosphatase. In comparing the high affinity phosphate transporters in *E. coli* and *S. meliloti*, the PstSCAB and PhoCDET systems. We find that, 1). They are both high affinity phosphate transport systems and induced in phosphate limited condition. 2). They are both in one operon. 3). *pstS* and *phoC* both have two *pho* boxs in their promoters and both are regulated by *phoB*. 4). *PhoCDET* mutant repress *orfA-pit* system. 5). Both *phoCDET* and *pstSCAB* mutant constitutively express alkaline phosphatase in Po and P2. 6). *PhoCDET* mutants lead *S. meliloti* synthesize DGTS constitutively at both low and high phosphate conditions. 7). *phoCDET* mutants grow very slow on both limited and excess phosphate medium (Bardin et al, 1996), it was most possible that cells division were stopped. Also it seems that *phoCDET* can sense the environment phosphate changes. According to the above comparisions, we address these questions: does *pstSCAB* system of *E. coli* function in place of *S. meliloti PhoCDET* system in free-living condition and in symbiosis? If the *S. meliloti phoCDET* system involved in *pho* regulation in the same way as *pstSCAB* system in *E. coli*? To answer these questions, we need to colne the *pstSCAB* system into vector which can replicate in *S. meliloti* and introduce into *phoCDET* mutants. Then test the growth of the new strains and most importantly, do plant test. What is the phenotype of this construction? Fix+ or Fix-?

In *Caulobacter crescentus*, *CcrM*, an adenine DNA methyltransferase, is an important target of the Lon protease pathway. The *ccrM* gene is transcribed only in the predivisional cell and rapidly degraded prior to cell division. The only presence of CcrM protein in the predivisional stage of the cell cycle results in cell-cycle-dependent variation of the DNA methylation state of the chromosome. A lon protease null mutant leads to constitutive expression of *ccrM* which results in a fully methylated chromosome throughout the cell cycle and would not degrade and thus contributes to several developmental defects including: 1). a frequent failure to complete cell division, 2). loss of precise cell-cycle control of initiation of DNA replication, 3). the formation of abnormally long stalks because cell growth and no division (Wright R, 1996). Also, In *Caulobacter crescentus*, stalk biosynthesis is regulated by cell cycle cues and by extracellular phosphate concentration. Phosphate-starved cells undergo dramatic stalk elongation to produce stalks as much as 30 times as long as those of cells growing in phosphate-rich medium. PhoB was required for stalk elongation in response to phosphate starvation. Both *pstS* and *phoB* mutants were deficient in phosphate transport. *pst* mutants made long stalks in both high- and low-phosphate media. *pstS* transcription is dependent on *phoB.* The signal transduction pathway that stimulates stalk elongation in response to phosphate starvation is mediated by the Pst proteins and the response regulator PhoB. (Gonnin M, 2000). It is very likely that in *Caulobacter crescentus, pstSCAB* system and Lon protease is involved in cell division and stalk elongation, but it is not know whether *pstSCAB* system involved in Lon protease expression and regulation or if they are related to each other?

PhoCDET mutations lead to constitutively Apase expression (Bardin & Finan, 1997) and EPSII but normal EPSI production (Oresnik, 1994). EPSII and Apase are

known to be induced under phosphate limited conditions (Zhan 1991). Also, nodules induced by *ndvF* mutants were empty with few bacteriod, the infection is blocked at an early stage and bacteria are not released from the infection thread, namely, they are Nod^+ and Fix^- (Charles, 1991). Recently, One *S. meliloti* Lon protease gene was investigated (Summers, 2000). Lon mutants lead to contitutively alkaline phosphatase expression both in phosphate excess and limited conditions. In addition to EPSI, the *lon* mutant also constitutively synthesized EPSII. Because the *phoCDET* mutant and *lon* mutant have the same phenotype: Apase+ and EPSII production, we would ask, if the *phoCDET* phenotype has any relationships with Lon protease expression and regulation?

Caulobacter crescentus cell can not divided lead to the elongation of the stalks. In *Escherichia coli*, mutants lacking the global regulatory protein integration host factor (IHF) show an increased level of alkaline phosphatase and a decreased level of Pst system. IHF binds weakly but specifically to the promoter region of the pst operon but does not bind to a fragment that includes the promoter region of *phoA*. It is proposed that IHF is a positive regulator of the *pst* operon and as such controls indirectly the expression of *phoA* (Spira B,1999). In *S. meliloti*, bacteroid differentiation occurs after the bacteria enter the cytoplasm of plant cells in an endocytic-like process. In indeterminate nodules, which are characterized by a persistent meristem, the bacteria are released into plants cells that have ceased dividing. Bacterial cell division occurs one or a few more times before differentiation. The differentiating bacteria enlarge to four to seven times the length of vegetative bacteria (Valerie Oke & Sharon R Long, 1999). The factors which affect *S. meliloti* cell division or elongtation most possibly affect *S. meliloti* bacteroid/nodule formation.

In *S. meliloti*, *phoCDET* mutants block infection at an early stage and few bacteria are released from the infection thread, that was most possibly because the cells can not divided. We may ask, if *phoCDET* is also involved in *S. meliloti* cell division and elongation? If *S. meliloti* change its longth under phosphate starvation? It is not difficult to identify whether *S. meliloti* alters its cell shape under phosphate starvation. Considering that 1). *PhoCDET* mutant represses *orfA-pit* system. 2). *PhoCDET* mutants were Apase$^+$ in both P_0 and P_2. 3), *phoCDET* mutants grow very slow in both P_0 and P_2 in free-living conditions. 4). *PhoCDET* mutants lead to increasing EPSII production (Oresnik IJ, 1994) and normal EPSI production (Charles TC, 1991). 5). *phoCDET* mutantion lead to constitutively synthesize DGTS in both low and high phosphate conditions (Geiger et al 1999). We believe it is possible that *phoCDET* is involved in phosphate regulation other than just phosphate transport. Hence, identification of the cell shape of *S. meliloti phoCDET* mutant and Lon protease mutant will help resolve this question. Also, in free-living *S. meliloti*, if the cell shape is different during growing in phosphate excess and limited medium?

2. phosphate regulation and bacteroid development and nodule formation.

In the symbiosis of *S. meliloti* and plant *Medicago sativa* (alfalfa), alfalfa nodule invasion by *S. meliloti* and successful establishment of a nitrogen-fixing symbiosis between the two partners must be mediated by any one of the three symbiotically important polysaccharides: succinoglycan (EPSI), EPS II, or K antigen (also referred to as KPS) (Pellock BJ. 2000). The synthesis and regulation of various polysaccharides in *S. meliloti* is a very complicated process. EPSI is the main product in normal *S. meliloti* cells and it is encoded by *exo* gene cluster located on the pEXO megaplasmid. EPSII is induced in phosphate limited conditions and is encoded by *exp* gene cluster, which are also located on the megaplasmid but were separated from *exo* gene cluster. *Exp* genes were up-regulated by MucS and down-regulated by ExpR and MucR and are positively regulated by PhoB. *Exo* genes are up-regulated by ExoS MucR and down-regulated by ExoR (Figure 23. Reviewed by Punita Aneja, PhD thesis). The reason for the production of two functional exopolysaccharides and how they are regulated were not clear. Low-phosphate conditions stimulate the production of EPS II in Rm1021. It was phosphate concentrations that determine which exopolysaccharide is produced by *S. meliloti*. The low-phosphate conditions normally found in the soil (1 to 10 μM) stimulate EPS II production, while the high-phosphate conditions inside the nodule (20 to 100 mM) block EPS II synthesis and induce the production of succinoglycan (EPSI). The EPS II produced by *S. meliloti* in low-phosphate conditions does not allow the invasion of alfalfa nodules. Perhaps this invasion phenotype is due to the lack of the active

molecular weight fraction of EPS II required for nodule invasion (Mendrygal KE, 2000).

In *phoCDET* mutants, the bacteria infection were stopped at the earlier stage, even when supplied with EPSII. Also, *phoCDET* mutants grow very slowly on limited and excess phosphate medium (Bardin SD, 1996). The reason was also not clear. *phoCDET* system, although it was positively regulated by PhoB itself, may be a signal components and involved in phosphate regulation. If we think about this issue together with cell cycle and cell division, we may hypothesis that, 1). In *phoCDET* mutants, the bacteria cell division is stopped, then the infection was stopped because the effective infection requires a large number of bacteria. 2). *PhoCDET* mutants block the signal transduction passways in the cell metabolism, which changes the regulation of EPSI or EPSII production. This hypothesis may help explain the above phenomena. To test the hypothesis, we need to measure the *exo* and *exp* genes expression in *phoCDET* mutant (*phoB* wildtype) background in phosphate limited and excess conditions, and also in free-living cells and in bacteroid (if it forms).

Synthesis and regulation of various polysaccharides in S. meliloti

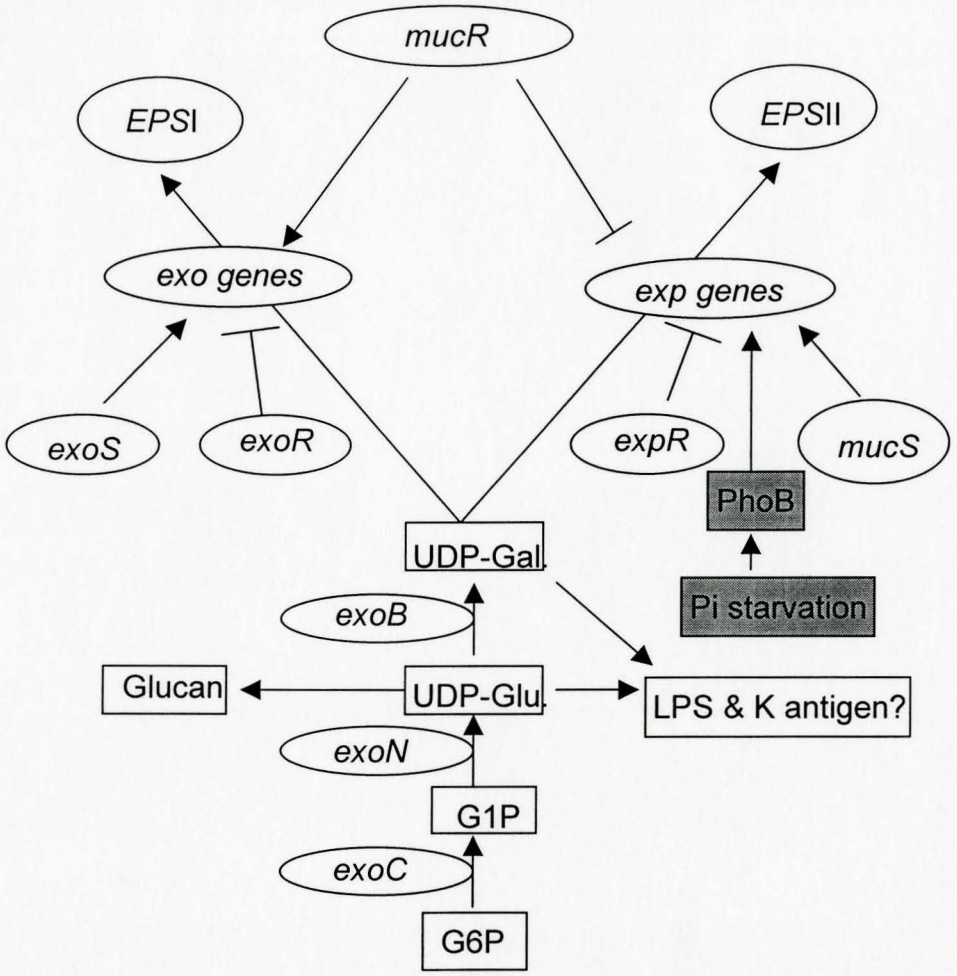


Figure 23, Synthesis and regulation of various polysaccharides in *S. meliloti,* → indicates positive regulation. ⊣ Indicates the negative regulation (reviewed by Punita Aneja, Ph.D. thesis , McGill University 1999)

3. Phosphate regulation and other function. Identification of other putative *pho* regulons in *S. meliloti*.

Phosphate regulation and its signal transduction involved in global metabolism and regulation in bacteria.

pho regulon may include genes unrelated with phosphate transport and uptake. Also PhoB may regulate genes unrelated with phosphate transport or uptake. Since phosphate is one of the important components of cell wall, it is very reasonable to presume that phosphate regulation play important role in soil bacteria metabolism, especially during the bacteria living in stress conditions, for example, nutrition stress, dry, salty, or acid environment.

In *E. coli*, phosphate regulation has been investigated intensively. *pst* mutation lead to an avirulent pathogenic strains to pigs (Lee 1989, Daigle 1995, Sinai 1993). Pst also involved in NAD synthesis (Hove-Jensen B, 1996) and production of the surface polysaccharides (Daigle et al, 1995). phosphate starvation induces PolyP accumulation and involves in bacterial motility, *rpoS* expression, cell competence and other functions (Geissdorfer et al, 1998. Rao et al, 1998). The *Escherichia coli* IciA potein specifically inhibits the initiation of chromosomal DNA replication (Han et al, 1999), and the *iciA* gene is a member of the *pho* regulon. *Escherichia coli phoB-phoR* regulatory system can sense an external acid variation and regulate transcription of the *asr* gene (designated *asr* for acid shock RNA) (Normark, S. 1999).

Phosphate regulation can also play a role in microbial virulence (Ostroff 1989). In *Agrobacterium tumefaciens* and *salmonella typhimurium*, low phosphate levels induced synthesis of virulence factors which direct a conjugation-like transfer of tumorigenic DNA from the bacterium to the nuclei of the infected plant genome

(Winans SC, 1990, Aoyama T 1991, Pan SQ 1993, Jin SG 1990, Winans SC 1994). *Vibrio cholerae pho* regulon involved in colonization ability and adaptation of *V. cholerae* to the intestinal environment (Ketley 1999, Wu H, 2000).

In *Bacillus subtilis* PhoP-PhoR two component system controlled the synthesis of cell wall anionic polymer, teichoic acid, and teichuronic acid (Ying Qi, 1998, Muller JP, 1997). Phospahe starvation also induces sigmaB dependent general stress regulon in *Bacillus subtilis* (Antelmann H, 2000). In *Caulobacter crescentus*, Pst and PhoB regulated the signal transduction pathway that control cell division and stalk elongation in response to phosphate starvation (Gonin M, 2000). In *Mycobacterium smegmatis,* Pst has the ability to promote drug efflux in addition to its involvement in phosphate transport (Bhatt K, 2000).

In *S. meliloti*, phosphate starvation leads to the expression of *exp* genes and EPSII production, which can function as a signal molecular in place of EPSI in the nodule invasion thread formation. But if phosphate regulation involved in metabolism, acid shock, heat shock, swimmimg or mobility, energy transfer and other stress response are still unknown. Recently, *S. meliloti* Tn5 mutants for fast swarming were isolated and found that they survived and grew as well as or better than the wild type under all of the circumstances tested. Exopolysaccharide (EPS) synthesis was reduced in each of the FS mutants when they were grown on defined succinate-nitrate medium. The spontaneous FS variants and Tn5 FS mutants were symbiotically effective and competitive in alfalfa nodulation, but the exact insertion site were not reported (Wei et al, 1999). During starvation (no available C, N, or phosphate), *S. meliloti* cells lost flagella (inactivation of flagellar motors) and flagellar integrity (Wei et al, 1998).

The genome identification of S. meliloti found 30% of gene with unknown function. Also large fragment (100kb) deletions on megaplasmid pSymb did not have affect on nitrogen fixation ability, but these test were done in labortory and large amount of bacteria were inoculated on test plant. They are different from the actual situations in field soil, in which the bacteria need to survive and compete with other bacteria for limited nutrients, and finally inoculation on host plant. We could not get rid of the possibility that these large deletion fragment contain genes involved in survival and competition in field soil. Considering the importance of phosphate to soil bacteria, it is very likely that some of these competition or stress induced genes are phosphate regulated.

Identify other *pho* regulon.

Because phosphate signal transduction have global effects on bacteria cell metabolism and regulation, it would be very useful to investigate these *pho* regulons. The *S. meliloti* international genome project will be finished in year 2001, many new genes in *S. meliloti* were found but with unknown function (30%). It is well known that prokaryotics organism are very economic in its inner structure and function, it is different from human or plants which have lots of unfunctional or silent genes. The question is how to identify all the phosphate regulated genes and their functions in *S. meliloti?*

There are several ways to investigate putative *pho* regulon and its function in *S. meliloti,* 1) perform *pho* box motif search using the computer progeam. 2). DNA microarrays the whole S. *meliloti* genome in the phosphate limited conditions. 3). Protein examination by two-dimensional gel electrophoresis. 4). Invididual gene expression in phosphate limiting and excess conditions. 5). PhoB binding and

regulation analysis. DNA microarrays have turned out a powerful method for global monitoring of gene expression in *Escherichia coli* (Richmond CS, 1999). The pattern of proteins synthesized in *Escherichia coli* upon limitation for Pi was also examined by two-dimensional gel electrophoresis. Some new genes with putative *pho* box consensus sequence in their promoter regions were found (Van Bogelen RA, 1996).

One very powerful approach is to use a *pho* box search program such as that developed by Dr Richard Morton in this department to help identify the putative phosphate regulation genes or *pho* regulons. Using the *pho* box motif search program, I searched the pSyMb AF region part 1 sequence which is about 140 kb and found some of the putative *pho* Boxes, one of them is located upstream of a open reading frame which has 61% homologous identities to probable amino-acid ABC transporter ATP-binding protein of Rhizobium sp NGR234 (Appendix I).

It is not difficult to search the whole *S. meliloti* genome (once the whole genome sequence is available) for the putative *pho* Boxs in this way. But to determine whether these putative *pho* Boxes are functional, further analysis to monitor the expression of the putative genes under phosphate excess and limited growth conditions and in a phoB vs wildtype background are necessary. Aslo, it would be very useful to overexpress and purify PhoB protein in order to measure PhoB binding to target region in virto (Sol M, 1998).

4. Cross-talk of phosphate regulation.

Sensor kinases and response regulators of two-component regulatory systems share extensive sequence similarities to many other family members, even among phylogenetically distant species. These sequence similarities probably lead to structural similarities that are responsible for cross-reactivities between sensor kinases and response regulators of different systems. This phenomenon, called "cross-talk," has been observed in vitro, and it has been implicated in complex phenotypes in vivo. In *E. coli*, the sensor kinase PhoR activates the response regulator PhoB that in turn activates synthesis of bacterial alkaline phosphatase in response to limiting phosphate levels. In the absence of PhoR, the sensor kinase CreC or acetyl phosphate activates PhoB (Figure 3). Furthermore, cross-talk interactions have been used to identify new two-component regulatory genes by complementation of known sensor kinase mutants. These results reinforce the concept that individual two-component systems share a common mechanism and suggest that it is likely that similar regions of the sensor kinases and response regulators are involved in the transmembrane signal sensing, autophosphorylation, and interprotein phosphorelay steps of these signal transduction pathways (Fisher SL, 1995, Wanner BL 1992).

In *Agrobacterium tumefaciens*, plant signals induce *A. tumefaciens* virulence genes through the VirA-VirG two-component regulatory system. The VirG protein is phosphorylated by the VirA protein and activate other vir gene expression(Jin SG, 1990, Winans SC. 1994). The *Agrobacterium* virG protein showed significant homology with the *Escherichia coli ompR, phoB* (Melchers LS, 1986). Besides plant signal, virulence genes are also induced by phosphate limitation. The response of the

virulence gene to phosphate limitation does not require the positive regulator VirG for the virulence regulon, but depended entirely on the presence of PhoB protein, the positive regulator for the phosphate regulon. Both VirG and E. coli PhoB can recognize the virulence gene promoter in vitro (Aoyama T, 1991). Also, *Agrobacterium tumefaciens chvI* gene which encoded a predicted protein with amino acid similarity to the family of bacterial response regulators and 35% identify to *E. coli* PhoB can complements an *Escherichia coli phoB* mutation and is required for virulence (Mantis NJ, 1993). These results indicate that cross-talk occurs between the *vir* regulon and *pho* regulon. But how these two regulatory system interacted is unknown.

In *Enterococcus faecium*, VanS is a two-component transmembrane sensory kinase, together with its response regulator VanR, activates the expression of genes responsible for vancomycin resistance. The cytoplasmic domain of VanS is capable of high level activation (> 500 fold) of the *Escherichia coli* response regulator PhoB in vivo in the absence of its signaling kinases PhoR, CreC (PhoM), or acetyl phosphate synthesis. The activation is due to efficient cross-talk between VanS and PhoB, phospho-VanS catalyzed transfer of its phosphoryl group to PhoB (Fisher et al, 1995, Haldimann et al, 1997, Silva et al, 1998).

In *S. meliloti*, the genes coding for acetate kinase (*ackA*) and *pta*, which codes for phosphotransacetylase are part of an operon (*orfA-pta-ackA-fabI*) and is up-regulated in response to phosphate limitation but required intact *phoB*. *pho* boxes were found in the phosphate starvation-inducible transcriptional start sites upstream of orfA. Mutations in either *ackA* alone or both *pta* and *ackA* do not affect the nodulation or nitrogen fixation phenotype of *S. meliloti* (Summers et al, 1999).

Whether *pta* and *ackA* are involved in phosphate regulation (activate phoB) is not clear. In this study, we interrupted *phoR* gene by *lacZ-Gm* cassette insertion and found the Apase phenotype is dependent on the direction of the insertion. Without functional *phoR*, there are must be other kinase which activate PhoB. Further study needed to address these questions.

5. **Overexpression of phosphate transport.**

M. tuberculosis has three *pstS* genes, *PstS-1, PstS-2*, and *PstS-3*. It was presumed that the mycobacterial gene duplications may be a subtle adaptation of intracellular pathogens to phosphate starvation in their alternating growth environments (Lefevre et al, 1997). Amplification of *Mycobacterium* phosphate specific transporter (Pst) generated ciprofloxacin resistant colony (CIPr) (Chakraborti PK, 1999). Also *Mycobacterium smegmatis pstB* amplification shows enhanced phosphate uptake and increased resistance to fluoroquinolones (Bhatt et al, 2000). In plant, expression of the *Arabidopsis* high-affinity phosphate transporter at increased levels in tobacco-cultured cells could increase the rate of phosphate uptake, the transgenic cells exhibited increased biomass production when the supply of phosphate was limited (Mitsukawa et al, 1997). Increasing the expression of phosphate transport systems seems to lead to series of phenotype changes in bacteria or plant cell. This may occur as a result of an increase in the cellular phosphate levels which help the bacteria survive. Second, if the phosphate transporter system itself is also involved in phosphate regulation or regulation of other pathways. It will become more complicated if it also influence the other signal transduction ways. There is evidence that *pstSCAB*, the high affinity phosphate

transport system in *E. coli* is involved in phosphate regulation. Therefore, It will be very interesting to know if overexpression of *S. meliloti* PhoCDET system, the high affinity phosphate transport system will have any effect on phosphate transport and regulation?

In soil, the phosphate concentration is around 1-10 μM, while in plant, 10-20mM and in the nodule 100mM (Mendrygal KE, 2000). Previous study (Bardin et al, 1997, 1998) shown that *phoCDET* is induced and function in low phosphate condition and repressed in high phosphate condition (2mM). *orfA-pit* system is induced in high phosphate condition (2mM) and repressed in low phosphate conditions. These data suggest that in root nodules the PhoCDET system is repressed and OrfA-pit system is functional. But what really is the situation in the nodule during symbiosis? Determination of the mRNA levels for *phoCDET* and *orfA-pit* in nodules will help to answer this question.

6. *pstS, pstC, pstA, pstB* and *phoR* function?

In bacteria the P_i signaling response involves three processes: activation, deactivation, and inhibition (Banerjee SK, 2000). In *E coli* the regulation of phosphate (*pho*) regulon by environmental inorganic phosphate (P_i) levels is a paradigm of a bacterial signal transduction pathway in which occupancy of a cell surface receptor (the P_i-specific binding protein PstS) regulates gene expression in the cytoplasm. This signal transduction pathway requires seven proteins including: (i) two members of the large family of two-component regulatory systems, response regulator PhoB (a transcriptional activator) and its partner, histidine sensor kinase PhoR (itself an integral-membrane protein); (ii) P_i-specific transport (Pst) machinery (PstS, PstA,

PstB, PstC); and (iii) PhoU, a negative regulator of unknown function. Activation of *E. coli Pho* regulon involves autophosphorylation of PhoR by ATP, phosphotransfer to PhoB, and transcriptional activation of *pho* regulon promoters by phospho-PhoB (P-PhoB). Inhibition prevents phosphorylation of PhoB when P_i is in excess; it requires all seven P_i signaling proteins (PhoB, PhoR, PhoU, PstA, PstB, PstC, and PstS) (Haldimann A, 1998).

In *S. meliloti*, previous researches have shown that PhoB positive regulates PhoCDET system and negative regulates o*rfA-pit* expression (Bradin & Finan, 1998, 1997). PhoB is required for *exp* and *pta-ackA* genes transcription (Summers et al, 1998; Chan 1994). Interestingly, mutations in the *phoCDET* genes resulted in a repression of *orfA-pit* transcription. Further it was found that *phoB* and *orfA-pit* double mutant could not be made. So, if there exists another phosphate transport system in *S. meliloti*? In this study we found that *pstB* mutant block the alkaline phosphatase expression. But this repression was due to the polar mutation in the operon and was *phoB*$^-$ phenotype. *E. coli* PstB protein is an ATPase, the PstB bound and hydrolyzed ATP, producing ADP (Chan et al, 1996). Here, we do not know exactly the function of pstB in *S. meliloti*. Our data shown that *pstA-pstB-phoU-phoB* form one operon and this operon is neither regulated by phosphate concentration nor regulated by PhoB protein. There appears to be no *pho* box in the putative promoter region of the *pstAB-phoUB* operon, but there is a very clear *pho* box upstream of the *pstS* gene, this is located at 111 bp relative to the translational start site of *pstS* open reading frame. Is this *pho* box really a PhoB binding site? Is *pstS* expression regulated by phosphate concentration? This question need to be examined further. In *E. coli*, PstS is presumed to function as a environmental phosphate senser. We do

not know what is the function of *pstSCAB* genes in *S. meliloti*? We subcloned the *phoR-pstS* gene in suicide vector for gene interruption investigation (Figure 18, pTH694). We would like to know the phenotype of *pstS* mutants? We have found that the phenotype of *phoR lacZ-Gm* cassette insertion mutations was dependent on the orientation of the *lacZ-Gm* insertion cassette (Figure 15). Considering the powerful *aaccI* promoter and the downstream *pstS* gene, it is most possible that, 1). *phoR* has effect in *pstS* gene expression. 2). The powerful Gm promoter has increase on *pstS* transcription. In view of the intergenic distance between *phoR* and *pstS* (200bp), and the presence of the *pho* box upstream of *pstS*, it appears unlikely that *phoR* and *pstS* are in one operon. Further analysis with the *ΩSp* insertion will be done to study *phoR* function. Whether *phoBR* forms two component system involved in phosphate regulation in *S. meliloti* need to be identified.

In addition, there is another gene in the upstream of *phoR* gene which encodes a protein with homogous identities of 49% (113/227) to PvdS protein in Mycobacterium tuberculosis. PvdS is an oxygen and iron dependent sigma factor in many bacteria which is induced in iron-limitation condition and involved in iron tansport (Xiong et al, 2000). PvdS is also the transcriptional activator of many genes invloved in virulence, pyoverdin (the fluorescent siderophore) biosynthesis and exotoxin A in *P. aeruginosa* (Leoni L, 2000). In *S. meliloti*, we have no idea about this *pvdS*-like protein and its function, especially it is interesting that it is so close to *phoR-pstSCAB-phoUB* gene fragment. Does PvdS has any role in phosphate signal transduction?

E coli Function genomics shown that *phoU* gene, but no *phoBR* gene, is expressed in minimal glucose medium other than in LB plus glucose (Han et al,

1999). Genome-wide expression profiling in *E coli* K-12 (Richmond et al, 1999) found that heat shock highly induced *phoBR, rpoD* and repress *pta* gene (phosphotransacetylase) expression. So in bacteria, *phoBR* definitely play a role in bacteria stress response, for example, heat shock. In *S. meliloti*, it was known that phosphate regulation EPSII production, but whether phosphate involved in bacteria survival or competition need to be identified.

In *E coli* phosphate starvation induced transcription of the (*pho*) regulon requires the specific activator protein PhoB in addition to the RNA polymerase holoenzyme containing the major sigma-factor sigma 70. PhoB promotes specific interaction between RNA polymerase and the *pho* promoters for transcriptional activation (Makino et al, 1993). In *S. meliloti*, Sigma 70 (Sigma A) factor was identified (Rushing et al, 1995). However we still do not know if Sigma 70 will interact with PhoB for *pho* regulon activation?

7. **Phosphate regulation and plant-bacteria signal transduction.**

Since phosphorus is required by both host plants and soil bacteria in symbiosis, but in soil, soluble inorganic phosphate (Pi) is very limited. Bacteria and plants utilize phosphate around roots, and plants therefore compete with microorganisms for available phosphate in the rhizosphere (Kragelund et al, 1997).

The nitrogen fixing bacterium *S meliloti* has evolved various mechanisms to adapt to phosphate limiting conditions. One is that, under phosphate-limited conditions, *S. meliloti* replaces its membrane phospholipids with lipids not containing phosphorus (Geiger et al, 1999). Another is that *S. meliloti* activates genes involved

in the high-affinity transport and assimilation of phosphate as well as other phosphorous-containing compounds.

To cope with decreasing Pi in the environment, plants have also evolved sophisticated developmental and metabolic adaptations to enhance Pi acquisition from the rhizosphere (Abel et al, 2000). Such strategies include, increased root growth, increased expression of Pi transporters (Liu et al, 1998) and alterations in metabolism, including secretion of acid phosphatases (Lefebvre et al, 1997) and RNAses (Bariola et al, 1994), which assist in the liberation of Pi from the rhizosphere. But at the molecular level, relatively little is known about the Pi-starvation response in plants and even less about its regulation.

Plant kinetic and molecular data show that there are at least two uptake systems for Pi across cellular membranes in plants, one with a high affinity and is either increased or de-repressed by Pi starvation, and one with a lower affinity and is constitutivly expressed (Schachtman et al, 1998). Phosphorus is acquired by plant roots primarily via the high-affinity inorganic phosphate (Pi) transporters. This transcripts for Pi transporters are highly inducible upon Pi starvation (Muchhal et al, 1999).

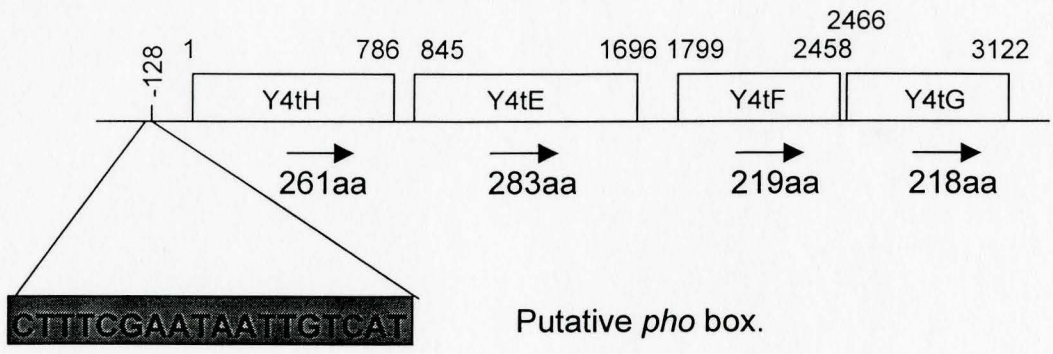
One high-affinity plant phosphate transporters (AtPT1 and AtPT2 genes) were cloned from phosphate-starved Arabidopsis thaliana roots, the transcripts of genes are expressed in roots and are not detectable in leaves. The steady-state level of their mRNAs increases in response to phosphate starvation (Muchhal et al, 1996). Recently, A cDNA (Mt4) from *Medicago truncatula*, which is induced in roots in response to Pi starvation was cloned. *Mt4*-like genes was also found in Arabidopsis and soybean (Burleigh et al, 1997 & 1999). Mt4 is the first cDNA reported to show

independent regulation by both phosphate and mycorrhizal fungi (Burleigh et al, 1998). Mt4 Expression was highly sensitive to exogenous applications of phosphate fertilizer; transcripts were abundant in roots fertilized with nutrient solution lacking phosphate, reduced when fertilized with 0.02 or 0.1mM phosphate and undetectable when fertilized with 1 or 5 mM phosphate. The Mt4 promoter region contains a conserved cis-element found in the promoters of phosphate starvation inducible genes of yeast and tomato. Also, another two cDNA clones (MtPT1 and MtPT2) encoding high-affinity phosphate transporters from a mycorrhizal root cDNA library (*Medicago truncatula/Glomus versiforme*) were identified. MtPT1 and MtPT2 transcripts are present in roots and transcript levels increase in response to phosphate starvation (Liu et al, 1998). The molecular mechanisms by which the shoot regulates Pi uptake are also unknown, especially during symbiotic conditions.

In symbiosis system between nitrogen fixation bacteria and host plants, we do not have any information about how plant and bacteria compete for limitted phosphate sources. Host plant supply C4 compounds such as Succinate, Malate and Fumarate to bacteria, bacteria supply fixed nitrogen to plant. But what is the final fate of phosphate after they are transported into bacteria? Most of the phosphate will go to metablism for bacteria to survive. The question is, will the bacteria also supply phosphate to host plant? Although phosphate is mainly acquired from the soil by Pi transporters located in the roots (Bieleske, 1973). Most vascular plants can acquire phosphate from the environment either directly, via the roots, or indirectly, via a fungal symbiont that invades the cortical cells of the root. In the symbiosis of host plant and arbuscular mycorrhizal, the mycorrhizal function as part of plant root for transport phosphate to plant from soil. Also the same plant signal molecular,

flavonoids, and the similiar mechanisms were used in both alfalfa mycorrhizae and *Rhizobium* induced nodule formation. In the two kinds of symbiosis, there exist energy and signal exchange between the host plant and microbials (Hirsch AM, 1998, Harrison MJ, 1998). We are wondering if the bacteriod or nodule, besides supplying fixed notrogen to host plant, also function as part of root system to acquire and supply phosphate for host plant?

Appendix 1. Gene encoding probably amino-acid ABC transporter ATP-binding protein and its putative *pho* box



CTTTCGAATAATTGTCAT — Putative *pho* box.

CTGTCATAAATCTGTCAT — *S. meliloti pho* box consensus.

CTGTCATAAATCTGTCAC
T A T — Compare with the *E. coli* *pho* box consensus:

→ indicate the direction of the gene open reading frame.

Y4tH: probable amino-acid ABC transporter ATP-binding protein of Rhizobium sp. NGR234. Length=257aa, Identities=156/255 (61%).

Y4tE: probable amino-acid ABC transporter ATP-binding protein of Rhizobium sp. NGR234. Length=300aa, Identities=92/284 (32%).

Y4tF: probable amino-acid ABC transporter ATP-binding protein of Rhizobium sp. NGR234. Length=238aa, Identities=102/217 (47%).

Y4tG: probable amino-acid ABC transporter ATP-binding protein of Rhizobium sp. NGR234. Length=218aa, Identities=73/158 (46%).

DNA sequence of S. meliloti *Y4tH*-like gene.

-146 -128

GCTGCTCGTCATTTGAGGCGAATGCTCAGCTTTTGAGCAC**CTTTCGAATAATTGTCAT**

GATATTATTATTCACATTGACATGATTTGGGAACCGGATCATCGTTAGGACATTAACGCAT

GGCGCCCAGAAGTGTGCAGCGGTTTGGAGCGACGCCATGCGCTTTAGTCTCAA

SD 0 →

AGGGAGAGGCGTGA**ATG**TCCCAGCCGATCATTCGCATCGACAACATCGTCAAGCG

ATACGGTCCCCTGACCGTGCTCGACGGCCTGTCGATGGAGGTGATGCCGGGCGAGA

AGCTGGCTCTCATCGGGCCGTCCGGTTCGGGTAAGACGACCATCCTTCGCATTCTGA

TGACGCTCGAGACCATCAGCGACGGTTTCATCCAGGTCGATGGCGAGCAGCTCTATC

ACATGAAGAAGGCGGGTAGCCTCGTCCCTGCCGACGAGCGGCATCTCCATAAAATGC

GCGAGAAGATCGGCATGGTCTTCCAGCATTTCAACCTGTTTCCTCATAAATGCGTTCTC

GACAACGTCACGCTGGCGCCGATGCTGACCAAGGGCATGGCGCGCGCTCAGGCGGA

AAAGCGCGCGATGGAGCTGCTCGACATGGTGGGCCTGGCCGACAAGGCCAAGAGCAT

GCCGGCACAGCTTTCCGGCGGGCAGAAGCAGCGCGTTGCGATCGCCCGGGCGCTGG

CGCTCTCACCCAAGATCATGCTTTTCGACGAGGTCACCTCCGCGCTCGATCCCGAGCT

TGTCGAGGAGGTCCTGAACGTCATGCGCAAGCTCGCGTCCGAGACGGACATGACCAT

GCTGCTCGTCACCCACGAGATGGGCTTTGCCCATGATTTCGCGGACCGGGTGCTTTTC

TTCGACCGCGGCAAGATCGTGGAGGAAGGAAAACCCGAGGACATTTTCCGGCATCCC

AAACAGGAGCGCACACAAACTTTCCTGCGCAAGATCATAGCGGCAGGGCACCGCGTC

TAAGCCTCACGGCCGACATTGCCTCAAGGAAAACATCGAGAGAACAGAGGAGTTGGG

AACGATGAAACTGAGAGATTTTATGGCAATGGCGGCAGGCGCCACCGCATTGATGGC

AGTTGCTGCGGCCACGC

Length=786bp, 262aa.

Appendix 2.

***phoR-pstS-pstC-pstA-pstB-phoU-phoB* genes Open Reading Frame BLAST-X search (the amino acid length and the corresponding homogous matches):**

(1). ***phoR***

1. phoR----Bacillus subtilis Length = 579 Identities = 95/236 (40%),
   89aa----324aa of ORF
   335aa---569aa of Bacillus subtilis phoR
2. phoR----Vibrio cholerae Length = 433 Identities = 111/323 (34%),
   3aa---322aa of ORF
   111aa---421aa of Vibrio cholerae phoR
3. phoR----Shigella dysenteriae Length = 431 Identities = 94/234 (40%),
   89aa—322aa of ORF
   188aa—417aa of Shigella dysenteriae phoR
4. phoR----Klebsiella pneumoniae Length = 431 Identities = 92/235 (39%),
   88aa---322aa of ORF
   187aa---417aa of Klebsiella pneumoniae phoR
5. phoR----Mycobacterium tuberculosis Length = 485 Identities = 85/225 (37%),
   102aa---324aa of ORF
   248aa—464aa of Mycobacterium tuberculosis phoR
6. phoR----Escherichia coli Length = 431 Identities = 95/234 (40%),
   89aa---322aa of ORF
   188aa---417aa of Escherichia coli phoR

(2). ***pstS***

1. possible periplasmic phosphate binding protein----Campylobacter jejuni
   Length = 331 Identities = 116/331 (35%),
   4aa---328aa of ORF
   1aa---304aa of Campylobacter jejuni pstS
2. phosphate ABC transporter, periplasmic phosphate-binding protein----Thermotoga maritima Length = 274 Identities = 82/280 (29%),
   11aa---288aa of ORF
   5aa---234aa of Thermotoga maritima pstS
3. phosphate-binding protein PstS homolog----Methanobacterium thermoautotrophicum Length = 278 Identities = 80/305 (26%),
   1aa---303aa of ORF
   6aa---253aa of Methanobacterium thermoautotrophicum pstS
4. phosphate ABC transporter, periplasmic phosphate-binding protein (pstS)----Borrelia burgdorferi Length = 280 Identities = 64/259 (24%)
   27aa---284aa of ORF
   29aa---241aa of Borrelia burgdorferi pstS
5. phosphate-binding protein--Pseudomonas putida Length = 334 Identities =70/312 (22%)
   4aa---291aa of ORF
   3aa---278aa of Pseudomonas putida pstS

(3). ***pstC***

1. putative phosphate transport system permease protein----Campylobacter jejuni
   Length = 304 Identities = 131/308 (42%),
   128aa---431aa of ORF
   4aa---301aa of Campylobacter jejuni pstC
2. pstC----Mycobacterium tuberculosis Length = 338 Identities = 95/310 (30%),
   137aa---427aa of ORF
   21aa---326aa of Mycobacterium tuberculosis pstC
3. putative PstC protein----Burkholderia sp. Length = 323 Identities = 86/296 (29%),
   130aa—415aa of ORF
   23aa---304aa of Burkholderia sp. pstC
4. peripheral membrane protein C----Enterobacter cloacae
   Length = 319 Identities = 87/300 (29%),
   137aa---426aa of ORF
   25aa---311aa of Enterobacter cloacae pstC
5. pstC2 ----Mycobacterium tuberculosis Length = 324 Identities = 90/299 (30%),
   133aa---421aa of ORF
   34aa----314aa of Mycobacterium tuberculosis pstC
6. high-affinity phosphate-specific transport system, cytoplasmic membrane component----Escherichia coli Length = 319 Identities = 78/287 (27%),
   137aa---414aa of ORF
   25aa----298aa of Escherichia coli pstC

(4). ***pstA***

1. putative phosphate transport system permease protein----Campylobacter jejuni
   Length = 362 Identities = 162/413 (39%),
   25aa---437aa of ORF
   6aa---358aa of Campylobacter jejuni pstA
2. a transmembrane component of the phosphate specific transport complex----Pseudomonas aeruginosa Length = 513 Identities = 65/182 (35%),
   217aa---382aa of ORF
   285aa---466aa of Pseudomonas aeruginosa pstA
3. pstA-1----Mycobacterium tuberculosis Length = 304 Identities = 76/228 (33%),
   215aa---439aa of ORF
   82aa----303aa of Mycobacterium tuberculosis pstA
4. integral membrane protein A----Enterobacter cloacae
   Length = 296 Identities = 67/211 (31%),
   217aa---427aa of ORF
   78aa----284aa of Enterobacter cloacae pstA
5. high-affinity phosphate-specific transport system, phosphate transport system permease protein pstA----Escherichia coli Length = 296 Identities = 67/214 (31%),
   214aa----427aa of ORF
   75aa----284aa of Escherichia coli pstA

(5). ***pstB***

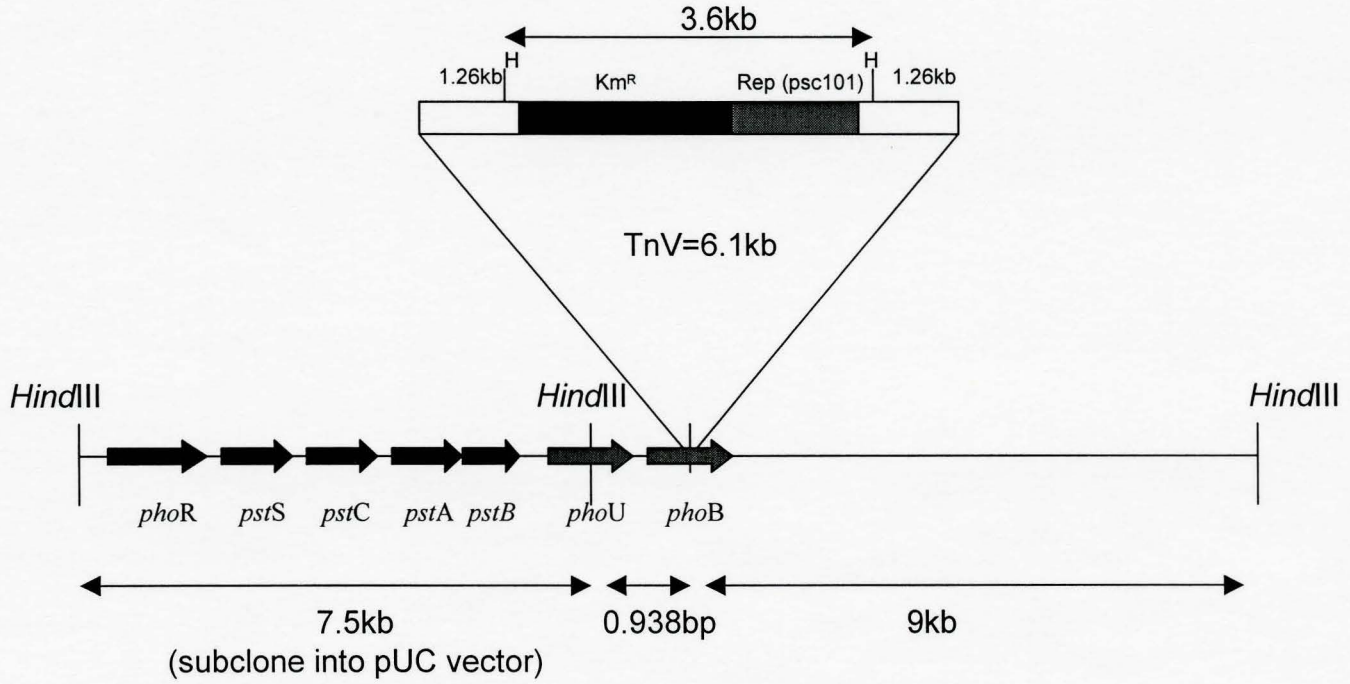
1. high affinity phosphate transport protein PstB----Caulobacter crescentus
   Length = 229 Identities = 171/229 (74%),
   43aa---271aa of ORF

1aa---229aa of Caulobacter crescentus pstB
2. peripheral membrane protein B----Pseudomonas putida
Length = 277 Identities = 159/243 (65%),
29aa---271aa of ORF
36aa---277aa of Pseudomonas putida pstB
3. alternate gene name: yzmE, similar to phosphate ABC transporter (ATP-binding protein)----Bacillus subtilis Length = 269 Identities = 145/244 (59%),
28aa---271aa of ORF
27aa---269aa of Bacillus subtilis pstB
4. peripheral membrane protein----Enterobacter cloacae
Length = 257 Identities = 141/255 (55%),
17aa---271aa of ORF
4aa---257aa of Enterobacter cloacae of pstB
5.. ATP-binding component of high-affinity phosphate-specific transport system ----Escherichia coli, Length = 257 Identities = 141/255 (55%),
17aa----271aa of ORF
77aa----257aa of Escherichia coli pstB
6. pstB----Mycobacterium tuberculosis Length = 276 Identities = 119/248 (47%),
24aa---271aa of ORF
22aa---267aa of Mycobacterium tuberculosis pstB

(6). ***phoU***
1. phosphate transport regulon regulator PhoU ----Caulobacter crescentus
Length = 230 Identities = 104/226 (46%)
34aa---259aa of ORF
4aa---229aa of Caulobacter crescentus phoU
2. a negative regulator of *pho* regulon----Pseudomonas aeruginosa
Length = 242 Identities = 80/212 (37%)
34aa---245aa of ORF
10aa---221aa of Pseudomonas aeruginosa phoU
3. phoU----Pseudomonas putida Length = 256 Identities = 79/212 (37%)
34aa---245aa of ORF
10aa---221aa of Pseudomonas putida phoU
4. negative regulatory protein of *pho* regulon----Enterobacter cloacae
Length = 241 Identities = 82/212 (38%)
34aa—245aa of ORF
9aa---219aa of Enterobacter cloacae phoU
5. peripheral membrane protein U -----Escherichia coli
negative regulator for *pho* regulon and putative enzyme in phosphate metabolism
Length = 241 Identities = 81/212 (38%)
34aa---245aa of ORF
9aa---219aa of Escherichia coli phoU

(7). ***PvdS***, a Sigma 54 factor involved in Fur regulon regulation, Fur regulon was responsible for the Iron transport and uptaking. pvdS----Mycobacterium tuberculosis. Length = 295 Identities = 113/227 (49%)
1232aa---552aa of pvdS ORF
28aa---254aa of Mycobacterium tuberculosis pvdS

Appendix 3. *phoR-phoB* region cloned from the R-plasmid carrying *phoB*::TnV.



According to this map, after selected the primer plasmid, DNA prepration and digestion with HindIII, the plasmid must produce one 7.5kb, 3.6kb and 2.3kb band, then it is supposed to be the right plasmid we wanted. Also, the cloned fragment was double check by sequencing or other approaches.

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