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Master of Science

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ABSTRACT

Phosphate Signaling Through Alternate Conformations of the PstSCAB Phosphate Transporter

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Phosphate is an essential compound for life. *Escherichia coli* employs a signal transduction pathway that controls the expression of genes that are required for the high-affinity acquisition of phosphate and the utilization of alternate sources of phosphorous. These genes are only expressed when environmental phosphate is limiting. The seven genes for this signaling pathway encode the two-component regulatory proteins PhoB and PhoR, as well as the high-affinity phosphate transporter PstSCAB and an auxiliary protein called PhoU. As the sensor kinase PhoR has no periplasmic sensory domain, the mechanism by which these cells sense environmental phosphate is not known. This paper explores the hypothesis that it is the alternating conformations of the PstSCAB transporter which are formed as part of the normal phosphate transport cycle that signal phosphate sufficiency or phosphate limitation. We tested two variants of PstB that are predicted to lock the protein in either of two conformations for their signaling output. We observed that the *pstB*Q160K mutant, predicted to reside in an inward facing, open conformation signaled phosphate sufficiency whereas the *pstB*E179Q mutant, predicted to reside in an outward facing, closed conformation signaled phosphate starvation. Neither mutant showed phosphate transport.

Keywords: phosphate homeostasis, two-component signal transduction, histidine kinase, ABC transporter

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Chapter 1 The phosphate regulation in *Escherichia coli*

1.1 Introduction/Background

Phosphorous is the fifth most abundant element of cells and though there are many sources of it, for *Escherichia coli* (*E. coli*), inorganic phosphate (P_i) is the preferred source. These elements are essential for energy metabolism, intracellular signaling, and they serve as an indispensible building blocks of several biomolecules. It is well known that cells alter their gene expression based upon the availability of environmental phosphate. The genes under control of phosphate regulatory system are called the Pho regulon. Pho regulon has been studied for many years (1,2), and the application of the basic knowledge of Pho regulation in the model organism *E.coli* is already helping to solve applied problems related to removal of phosphate from wastewater and for the decontamination of uranium sites (3,4). It has been also observed that phosphate-regulated genes are involved in the virulence of various pathogens (5). Therefore, better understanding of the phosphate regulation can help create drugs for certain diseases. Moreover, this study helps to resolve our understanding of the basic molecular mechanisms by which cells sense environmental phosphate and how they process that information to alter a cell's transcriptional machinery.

1.2 The Pho-regulon

The main purpose of the Pho regulon is to maintain optimal P_i levels inside the cell. To achieve this purpose the Pho regulon regulates the expression of many genes (6,7). One of these gene products is used as a reporter for our system – it is the periplasmic enzyme alkaline phosphatase, *pho*A (6,7). Also, *pst*SCAB, *pho*B and *pho*R are other important genes that are

under the control of Pho regulon which plays a vital role in sensing external Pi and its import into cells. The Pho regulon is controlled by a two-component regulatory system composed of the histidine kinase PhoR and phosphorylatable transcription factor PhoB (8,12). Also, a phosphate specific transporter (Pst) called PstSCAB plays a vital role in the uptake of P_i, and PhoU acts as a signaling molecule between the PhoR and PstB proteins (9,10). It is thought that PstSCAB senses environmental P_i and when P_i levels are low, it sends a signal thorough PhoU to PhoR, which then donates phosphate to PhoB (11). As a result, phospho-PhoB turns on transcription of P_i scavenging genes (see Fig 1).

When P_i is abundant, the vast majority of P_i enters the cell through two low-affinity, high velocity secondary transporters called PitA and PitB. Under these growth conditions a signal is sent through PstSCAB and PhoU to PhoR, which then functions as a phosphatase to remove phosphoryl groups from PhoB and turn off the Pho regulon.

1.3 The two-component system (PhoR and PhoB)

Hundreds of two-component proteins have been identified among different bacterial species that are necessary for cells to recognize and adapt to external signals (13). These two-component regulatory pathways are necessary throughout different stages of a cell's life such as its development, survival in various environmental conditions and adapting to stress. The mechanistic features of these systems are mainly composed of histidine kinase receptors and response regulators. The histidine kinase receptors recognize the external signals and relay them to the response regulators that are inside the cell, which in turn, gives a specific output such as acting as transcriptional activators to turn on the expression of certain genes. These histidine kinases and response regulators signal to each other through phospho-transferase activities.

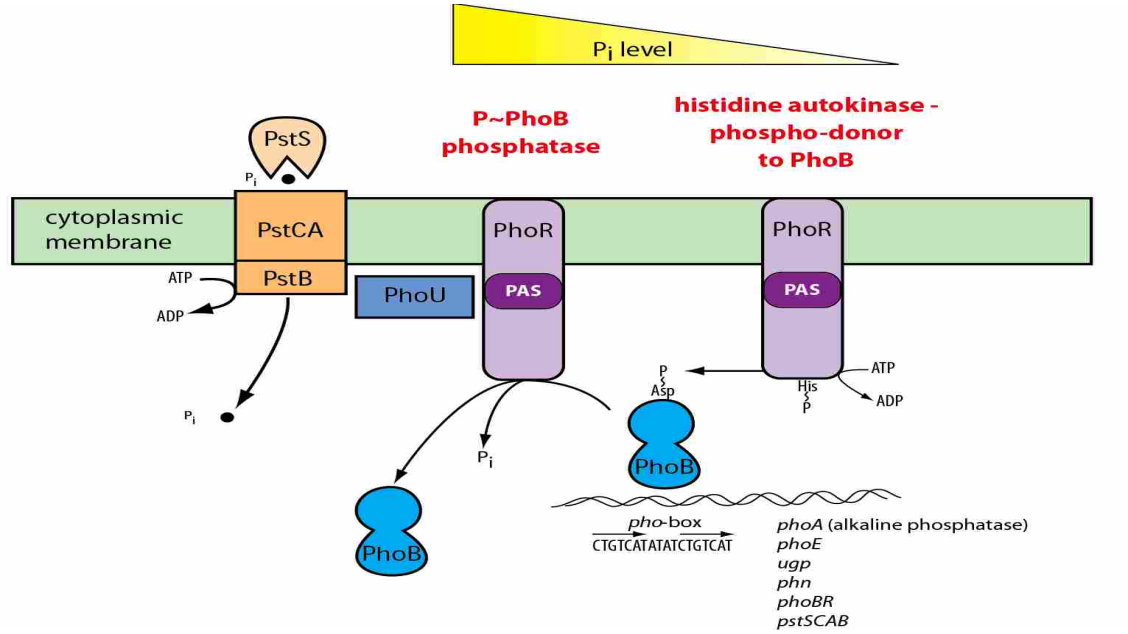


Figure 1. The Pho regulatory system.The two-component system (PhoR and PhoB) plays a vital role in Pho regulon by regulating the expression of P_i scavenging genes. In addition, Phosphate specific transporter (PstSCAB**),** interacts with the PhoU protein to control the PhoR's phosphatase and kinase activity. (Model from the McCleary lab)

1.3.1 PhoR

PhoR acts as a sensor histidine autokinase/phospho- PhoB phosphatase for the PhoBR two-component signal transduction system (14-16). It is categorized as a class I histidine kinase and it contains an N-terminal membrane domain, a PAS Per, ARNT and Sim domain, a DHp domain, and a C-terminal CA (catalytic and ATP binding) domain. PAS domains function in signal perception in a wide variety of organisms with its name being derived from the *Drosophila* proteins PAS (17,18). The DHp domain contains a conserved histidine residue which auto-phosphorylates under low P_i conditions and donates phosphate to the PhoB protein (19). This phosphorylated PhoB protein is then involved in elevating the Pho regulon expression for cells to

survive under low P_i conditions. Under P_i rich environments, PhoR acts as a phospho-PhoB phosphatase (phosphate mode), repressing the Pho-regulon genes to conserve cell energy and resources.

Studies on PhoR have shown that its N-terminal region is bound to the cytoplasmic membrane and its C-terminal end is the catalytic site of the protein (20). By performing experiments with various *pho*R deletion mutants, PhoR's role as a phospho-PhoB phosphatase has been confirmed and the predicted PhoR structure was shown (21). Mutant PhoR strains displayed constitutive expression of Pho regulon which indicates its necessity in the control of the Pho regulon. However, it is still unclear how PhoR receives a signal regarding the external P_i concentrations to regulate Pho expression. It lacks a significant periplasmic domain that could detect the external P_i levels.

1.3.2 PhoB

The PhoB protein is the response regulator for this two-component system. When it receives the phosphate group from PhoR, it binds to conserved DNA sequences that are located upstream of regulated genes and then interacts with RNA polymerase to activate transcription of genes which includes *pho*R, *pho*B, *pst*SCAB, *pho*U, *pho*A and other genes that are responsible for alternative phosphate sources (22-27). When phosphate is removed from PhoB, expression of these genes is blocked. Considerable work has been done on the PhoB protein and its crystal structure was solved by Blanco et al (27). PhoB is a heart shaped protein with an N-terminal receiver domain with a length of a 124-amino acid residues and a 99-amino acid long C-terminal effector domain. Moreover, the structures of DNA binding /transactivation domain and receiver/dimerization domain of PhoB were also solved (23). It was observed that in addition to

PhoR, acetyl phosphate and CreC proteins also phosphorylate PhoB but only in the absence of PhoR (28,29). This discovery has speculated a possible mechanism for cross talk of the Pho regulon with different regulatory systems (30).

1.4 PhoU

PhoU is an outer membrane protein that is needed for signal transduction in the Pho regulon. Under high Pi conditions, PhoU controls the excess import of Pi into the cells (31). Deletion mutations of *pho*U prevent signal transduction and lead to overexpression of Pho regulon and poor cell growth which underscores its importance in the Pho regulon. Recent work confirms PhoU's role in signal transduction of Pho regulon (32). Here, they used the Bacterial two-hybrid (BACTH) system to identify and characterize PhoU's interaction with PhoR. The PstSCAB complex senses environmental P_i and it passes this signal to PhoR through PhoU. Its interaction with PhoR, PstB and metals are important in forming a phosphate signaling complex at the membrane.

Multiple crystal structures of PhoU proteins from different organisms have been solved (34-36). It is mainly composed of two symmetric, three alpha-helix bundles and if often associates with metal ions. Recent work with *pho*U from *E.coli* found that metal binding is important for proper function of *pho*U (32). These same researchers later used protein modeling and docking methods to prove a possible mechanism in which PhoR 's phosphatase/kinase activity was mediated by PhoU (33).

1.5 PstSCAB transporter – an ABC transporter

In addition to the two-component system and PhoU, the phosphate-specific transporter (PstSCAB) plays a very important role in Pho regulon expression. Its primary role is to sense and import phosphate into the cell. Because PhoR does not have a periplasmic sensory domain, it has been proposed that the PstSCAB transporter is the ultimate sensor of environmental phosphate (37). However, it is still unclear how it communicates to PhoR regarding the P_i levels. In high external phosphate conditions, the PstSCAB transporter negatively regulates the kinase activity of PhoR by stimulating its phospho-PhoB phosphatase activity. Whereas, in low external phosphate conditions, this phospho-phoB activity is inhibited and autokinase activity of PhoR is turned on. Also, deletion mutations of any one of these transporter genes causes overexpression of Pho regulon and prevents signal transduction.

PstSCAB belongs to the ATP-binding cassette (ABC) transporter superfamily (38). These types of transporters are common among various organisms like bacteria, archaea and eukaryotes and are primarily involved in importing and exporting of molecules such as lipids or metabolic products across the plasma membrane (39). These transporters can be associated with the development of multi-drug resistance and other genetic diseases. The energy required for these transporters to function is met by binding and hydrolysis of ATP. These transporters have two subunits in common, one is the dimeric transmembrane domain (TMD) that contains the ligand-binding site and a dimeric nucleotide-binding domain (NBD) where ATP binds and hydrolyzes. Substrate binding protein is found in most organisms where a peripheral protein recognizes,

binds and then presents the substrate to the transmembrane domain. The PstS of the PstSCAB is the substrate binding protein.

The TMD is a channel through which the recognized substrate moves in to the cell. Twelve highly conserved α-helices that span the cell membrane forms the TMD (57,58). In the PstSCAB transporter, the PstCA corresponds to the TMD that has the ligand-binding site. The NBD is a protein dimer that contains several highly conserved structural motifs that are required for function. The Walker A motif, the Walker B motif and the ABC signature motif are some crucial protein regions that are responsible for ATP binding, ATP hydrolysis and stabilizing the bound ATP, respectively. (42,43,44). In the PstSCAB transporter, the PstB dimer corresponds to the NBD. A substrate bound to an outward-facing, ATP-bound transporter triggers the ATP hydrolysis. This ATP hydrolysis shifts the outward facing conformation to the inward facing conformation that leads to release of substrate into the cytoplasm.

Gardner et al. have recently showed that PhoU acts as an intermediate protein that interacts with the PstB protein of the PstSCAB transporter and with PhoR to modulate PhoR's kinase and phosphatase activities (32). One possibility of how the Pst transporter controls the partitioning of the kinase and phosphatase activities of PhoR could be by controlling the intracellular phosphate concentration, which could be sensed by the cytoplasmic PAS domain of PhoR. However, ^{31}P nuclear magnetic resonance studies have shown that intracellular P_i levels remain constant irrespective of the signaling status of the Pho regulon (51), suggesting that the intracellular P_i level is not responsible for the signal to PhoR from the Pst transporter. Another possibility that has been suggested is that PhoR senses the transport activity of PstSCAB (42). Countering this proposal are genetic studies of PstC and PstA that separated the transport activity of the protein

from its signaling activity (43,64). When Arg220 of PstA and Arg237 and Glu240 of PstC were mutated to glutamine residues, P_i transport was blocked but P_i signaling still occurred.

Considerable work on the homologous maltose transporter has suggested a refinement to the activity-sensing hypothesis to explain P_i signaling. The maltose ABC transporter from *E. coli* is a model to study the structure and mechanisms of ABC transporters (65,66,44,41). Its periplasmic binding protein is MalE, its TMD is made of MalF and MalG, and its NBD is MalK. Studies have shown that in the maltose transporter, the conformational changes triggered in the NBD domain are essential for the substrate transport across the membrane (40,41). Researchers found that Q140K and E159Q mutations in the NBD locked the transporter in the open and closed conformations respectively (see Fig 2). They used cross linkers of different lengths to probe the conformational states of the MalK dimer. These experiments clearly showed that the Q140K mutation caused the transporter to be in the open form, while the E159Q mutation was locked in the closed form. In addition, the crystal structure of the entire maltose transporter with the E159Q mutation was solved, showing conclusively that it was in the closed conformation (40).

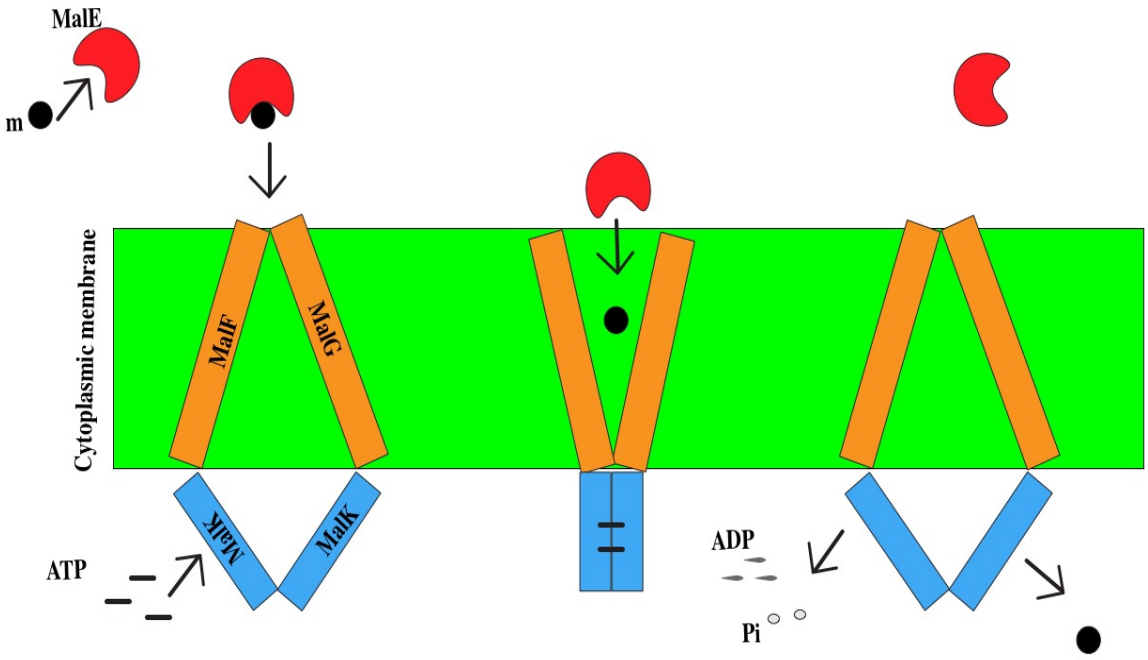


Figure 2. Model of maltose transport. Unbound and maltose-loaded MalE as well as dimerized MalK in the open and closed (ATP bound) state.

1.6 Conclusions

The molecular mechanisms that *Escherichia coli* uses to sense environmental inorganic phosphate (P_i) and how they respond to various external P_i levels has been studied for decades. Because P_i is essential for growth, optimum levels of it should be maintained intracellularly and to uptake P_i, *E.coli* employs either the low affinity P_i transporters (PitA, PitB) or a high affinity P_i transporter such as PstSCAB. This PstSCAB transporter, together with PhoU protein and a two-component regulatory system called PhoBR are absolutely necessary for P_i signal transduction, since mutations in any one of these seven genes leads to expression of Pho regulon irrespective of the external P_i levels. Though previous research identified the PhoBR two-component system as central in controlling the Pho-regulon, it remained unclear how PhoR sensed the phosphate levels.

Further research on the Pho regulon is necessary to understand the complete molecular mechanism through which phosphate is sensed and imported into the cell. The effects of the Pho regulon and its expression is associated with cell growth and virulence of various pathogens. Thus, knowledge on Pho regulon can potentially shed light in medicine and other many fields.

Chapter 2 Phosphate signaling through alternate conformations of the PstSCAB phosphate transporter

2.1 Abstract

Phosphate is an essential compound for life. *Escherichia coli* employs a signal transduction pathway that controls the expression of genes that are required for the high-affinity acquisition of phosphate and the utilization of alternate sources of phosphorous. These genes are only expressed when environmental phosphate is limiting. The seven genes for this signaling pathway encode the two-component regulatory proteins PhoB and PhoR, as well as the high-affinity phosphate transporter PstSCAB and an auxiliary protein called PhoU. As the sensor kinase PhoR has no periplasmic sensory domain, the mechanism by which these cells sense environmental phosphate is not known. This paper explores the hypothesis that it is the alternating conformations of the PstSCAB transporter which are formed as part of the normal phosphate transport cycle that signal phosphate sufficiency or phosphate limitation. We tested two variants of PstB that are predicted to lock the protein in either of two conformations for their signaling output. We observed that the *pstB*Q160K mutant, predicted to reside in an inward facing, open conformation signaled phosphate sufficiency whereas the *pstB*E179Q mutant, predicted to reside in an outward facing, closed conformation signaled phosphate starvation. Neither mutant showed phosphate transport.

2.2 Introduction

Inorganic phosphate is an essential compound for a cell's energy metabolism and is a component of nucleic acids, phospholipids and other cell constituents. Bacterial cells must maintain intracellular phosphate pools for optimal growth and they have developed intricate strategies to sense phosphate and control the expression of genes to best fit their environmental circumstances. The general principles underlying how these simple cells alter their gene expression based upon the availability of environmental phosphate are known (12,37,55,56). However, some of the molecular mechanisms by which cells sense phosphate and how they process that information to alter a cell's transcription machinery are not yet fully understood.

The genes under control of the phosphate regulatory system are called the Pho regulon. These genes are positively regulated in response to limiting external phosphate levels and include *phoA,* the gene for the periplasmic enzyme alkaline phosphatase (AP) that is often used as a reporter of the signaling status of the regulon (6,7). The PhoBR two-component system plays a central role in controlling the Pho regulon (8,14). PhoR is the sensor histidine kinase/phospho-PhoB phosphatase (14,16). It consists of an N-terminal membrane domain, a PAS domain, a DHp domain, and a C-terminal CA domain. Under phosphate-limiting conditions it phosphorylates itself on a conserved histidine residue and serves as a phospho-donor to PhoB. In phosphate-rich environments PhoR dephosphorylates PhoB by employing its phosphatase activity. It remains unclear how PhoR perceives external phosphate to regulate Pho regulon expression because it lacks a significant periplasmic domain that could bind phosphate. PhoB is the response regulator of this two-component system that binds to DNA when phosphorylated to interact with RNA polymerase and activate transcription (22,23,25). In addition to PhoR and PhoB, the phosphate-specific transporter PstSCAB and the PhoU protein are required for

phosphate signal transduction (28). It has been proposed that the Pst transporter is the ultimate sensor of external phosphate levels (37). Each of these seven genes is also part of the Pho regulon, which creates a positive feedback loop to amplify the phosphate starvation signal.

Mutations in any one of the transporter genes or *phoU* lead to overexpression of the Pho regulon, which shows that the default state of the signaling pathway is in a "kinase-on" mode (12). Phosphate signaling therefore involves the activation of the phosphatase function of PhoR. In high-phosphate growth environments the PstSCAB transporter and PhoU negatively regulate the kinase activity of PhoR and stimulate its phospho-PhoB phosphatase activity. In low-phosphate conditions, the kinase activity is stimulated and the phosphatase activity is inhibited.

PstSCAB is a member of the ATP-binding cassette (ABC) transporter superfamily (57,58). These transporters are widespread in nature and can serve as importers or exporters (59-61). ATP binding and hydrolysis power the transport of substances across the membrane through an alternating access mechanism in which a substrate is bound to an outward-facing, ATP-bound transporter, which then triggers the ATP hydrolysis that leads to the adoption of an inward-facing conformation and the release of the substrate into the cytoplasm. PstSCAB belongs to a class of importers that consists of an extracellular ligand binding protein, a transmembrane domain (TMD) that forms the channel through which the substrate will pass and a dimeric nucleotide-binding domain (NBD) where ATP is bound and hydrolyzed (62). In the Pst transporter, PstS is the periplasmic phosphate binding protein, PstC and PstA make up the TMD and PstB is the NBD that powers transport. The NBD domain includes several highly-conserved motifs that are required for function. For example, the Walker A motif, the Walker B motif and the ABC signature motif are important regions of the protein that are responsible for ATP binding, ATP hydrolysis and stabilizing the bound ATP, respectively (61,63).

Recent studies have shown that PhoU directly interacts with PstB and PhoR, suggesting a signaling mechanism in which PhoU binds to the PstB component of the PstSCAB transporter and relays environmental phosphate levels to PhoR, which then modulates its kinase and phosphatase activities (32,33). One possibility of how the Pst transporter controls the partitioning of the kinase and phosphatase activities of PhoR could be by controlling the intracellular phosphate concentration, which could be sensed by the cytoplasmic PAS domain of PhoR. However, ^{31}P nuclear magnetic resonance studies have shown that intracellular phosphate levels remain constant irrespective of the signaling status of the Pho regulon (51). So, if intracellular phosphate levels are not responsible for the signal to PhoR from the Pst transporter, then another possibility that has been suggested is that PhoR senses the transport activity (or movement) of PstSCAB (42). Countering this proposal are genetic studies of PstC and PstA that separated the transport activity of the protein from its signaling activity. Cox *et al.* found that when Arg220 of PstA and Arg237 and Glu240 of PstC were mutated to glutamine residues that phosphate transport was blocked, but that signaling still occurred (43,64). If non-functional transporters have static structures, which is a reasonable assumption, then phosphate-signaling can be separated from the dynamic motions involved in phosphate transport.

Considerable work on the homologous maltose transporter has suggested an alternative hypothesis to explain phosphate signaling. The maltose ABC transporter from *E. coli* is a model to study the structure and mechanisms of ABC transporters (65,66,41,44). Its periplasmic binding protein is MalE, its TBD is made of MalF and MalG, and its NBD is MalK. Studies have shown that the conformational changes triggered in the NDB domain by binding and hydrolyzing ATP are essential for substrate transport across the membrane. Daus et al. found that a *malK*Q140K mutant, which contains a mutation in the ABC signature motif and *malK*E159Q,

which contains a mutation in the Walker B motif are locked in the open and closed conformations, respectively (44). They used cross-linkers of different lengths to probe the conformational states of the MalK dimer in these mutants. These experiments clearly showed that the Q140K mutation caused the transporter to stably reside in the open form, while the E159Q mutant was locked in the closed conformation. Moreover, the crystal structure of the entire maltose transporter with the E159Q mutation was solved by Oldham et al. showing conclusively that it crystallized in the closed conformation (41). Based upon these studies, we hypothesize that there are two signaling states of the PstSCAB transporter that correspond to the two conformational states of the transport cycle; an outward facing, PstB-closed form and an inward facing, PstB-open conformation (See Fig 3). In the outward conformation, ATP is tightly bound across the PstB dimer interface. This form is present when phosphate levels are low and it promotes the "kinase-on/phosphatase-off" state of PhoR. Alternatively, the inward-facing conformation can only be reached following ATP hydrolysis when phosphate is present and is being transported. This conformation of the transporter promotes the "phosphatase-on/kinase-off" form of PhoR. When phosphate-bound PstS binds to the PstC/PstA transmembrane proteins, it triggers ATP hydrolysis within PstB and causes the conformational change that releases phosphate into the cytosol. Our hypothesis is that the complex of PhoU and PhoR recognize the alternate conformations of the PstSCAB transporter to modulate PhoR's alternate activities. In this paper, we test this model by creating *pstB* mutations that favor stable inward-facing and outward-facing conformations of the transporter and then assay the signaling status of the system.

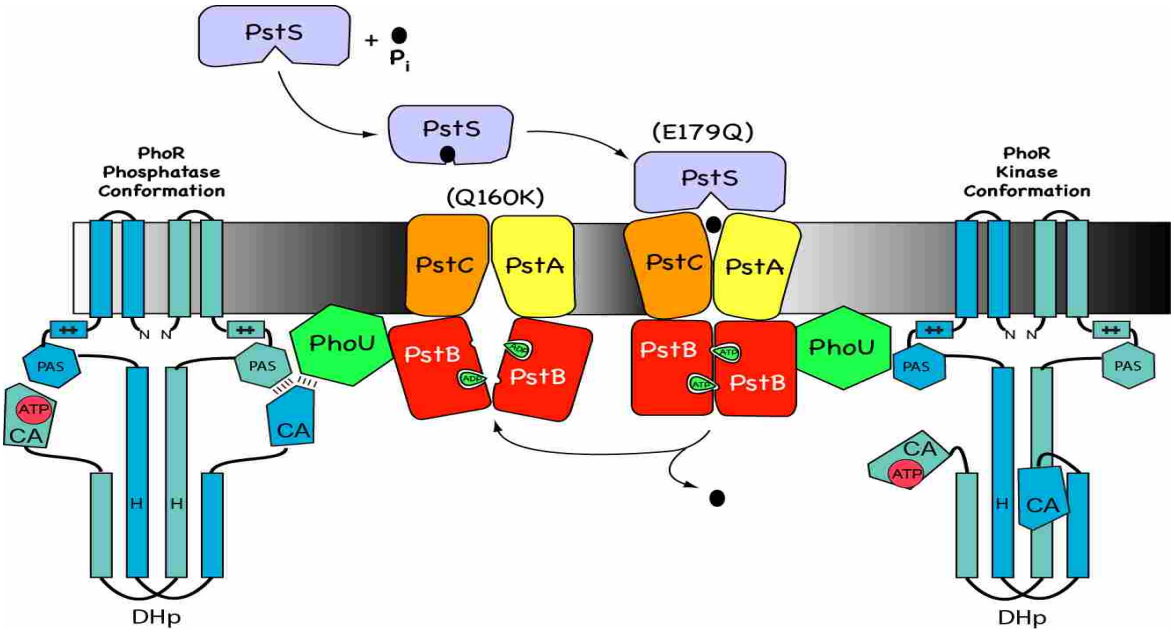


Figure 3. Conformational signaling model for control of the Pho regulon. The Q160K and E179Q mutations of the PstB, possibly lock the transporter in the inward-facing, PstB open and outward- facing, PstB closed conformations, respectively. (Model from the McCleary lab)

2.3 Materials and methods

2.3.1 Media

Cultures were grown in MOPS (morpholinepropanesulfonic acid) low phosphate and MOPS high phosphate minimal media (46). Low phosphate MOPS medium was prepared with 0.04% glucose (20% stock solution) and 0.1 mM phosphate (132mM K_2HPO_4 stock solution) and 1 X concentration of MOPS mixture. High phosphate MOPS medium was prepared with 0.06% glucose (20% stock solution) and 2 mM phosphate (132mM K_2HPO_4 stock solution) and 1 X concentration of MOPS mixture. MOPS no phosphate medium was prepared with 0.04% glucose (20% stock solution) and 1 X concentration of MOPS mixture. The antibiotic ampicillin was use at 50 μg/ml. All the three MOPS media were sterilized by filtration. LB agar plates were used for culturing strains (1 liter LB broth with 14 grams of agar and ampicillin when needed).

2.3.2 Strains, Plasmids and Growth conditions

E. coli BW25113 was used as the wild-type strain (50). Strain BW26337 contains a ΔpstSCAB-phoU::FRT mutation (50). The parent plasmids used in this study is pRR48 (45). pRR48 is a medium copy number plasmid that confers ampicillin resistance and expresses cloned genes from a Tac promoter (see Fig 4). p48SCABU plasmid was constructed by amplifying the pstSCAB-phoU operon by PCR using primers that contained embedded NdeI and KpnI restriction sites (see Fig. 5). Following PCR, amplified DNA was cleaned with Quick-Change purification kit and the PCR product was digested with NdeI and KpnI restriction enzymes and ligated into a similarly digested pRR48 plasmid vector. The plasmids encoding mutant versions of *pst*B were p48SCAB(Q160K)U, p48SCAB(E179Q)U (see Table 1 for description). They are derived from p48SCABU and were constructed using the Quick-Change site-directed mutagenesis kit from Agilent Technologies. Here, p48SCABU plasmid was amplified through PCR using two sets of forward and reverse primers that were designed to create point mutations to the 160th amino acid and 179th amino acid of PstB (see Table 2). These mutations were done separately. The mutant sequences were verified by DNA sequence analysis at the BYU DNA Sequencing Center. Strains were grown at 37°C with shaking.

2.3.3 Alkaline phosphatase assays

Cultures were grown overnight in MOPS LoPi medium at 37°C with shaking. 20 µl of overnight cultures were used to inoculate 2 ml MOPS HiPi or MOPS LoPi media and allowed to grow on a roller drum for 7 hours at 37°C. 600µl of these cultures were pelleted and then re-suspended in 600 µl of 1 M Tris-HCl pH 8.2. 100 µl of the cell suspension were then added to 100 µl of 1 M Tris-HCl pH 8.2 for a 1:2 dilution and the OD600 values were determined with a Thermo Scientific Multiscan FC 96 well plate reader. To the remaining 500 µl of cells, 10 µl of

0.1% sodium dodecyl sulfate and 20 µl of chloroform were added and the tubes were vortexed twice for 5 seconds at 20 second intervals. 50 µl of each sample were loaded into a 96 well plate containing 150 µl of 1 M Tris-HCl pH 8.2 and incubated at 37°C for 10 minutes to equilibrate the temperature. Following the incubation, 40 µl of 20 mM p-nitrophenyl phosphate in 1 M Tris-HCl pH 8.2 were added and OD420 values were determined at 1 minute intervals for 20 minutes and the maximum kinetic rates for each sample were measured (ΔA420/min). Finally, arbitrary AP units were calculated as (1000 × maximum kinetic rate)/(2 × OD600 of the overnight culture). Each strain was assayed using two biological replicates in duplicate. The average AP values of each sample with error bars representing standard deviations were reported.

2.3.4 Measurement of phosphate depletion and phosphate-signaling during a growth curve

Cells were grown overnight in MOPS HiPi medium at 37° C with shaking, pelleted and re-suspended in MOPS minimal medium with 0.06% glucose without phosphate. The re-suspended cells were then inoculated into flasks containing 40 ml of MOPS minimal media containing 60 µM phosphate and 0.4% glucose to a starting OD600 of 0.02 and grown at 37°C with shaking. 2 ml of cells were collected at hourly intervals of which 1 ml was used to measure the OD600 and the other 1 ml of cells were pelleted by centrifugation. The supernatant and pellets were separated and stored at -20°C for AP and phosphate assays. The supernatant's phosphate concentrations were quantified using the commercially available Malachite Green Phosphate Assay Kit by BioAssay Systems as directed by the manufacturer.

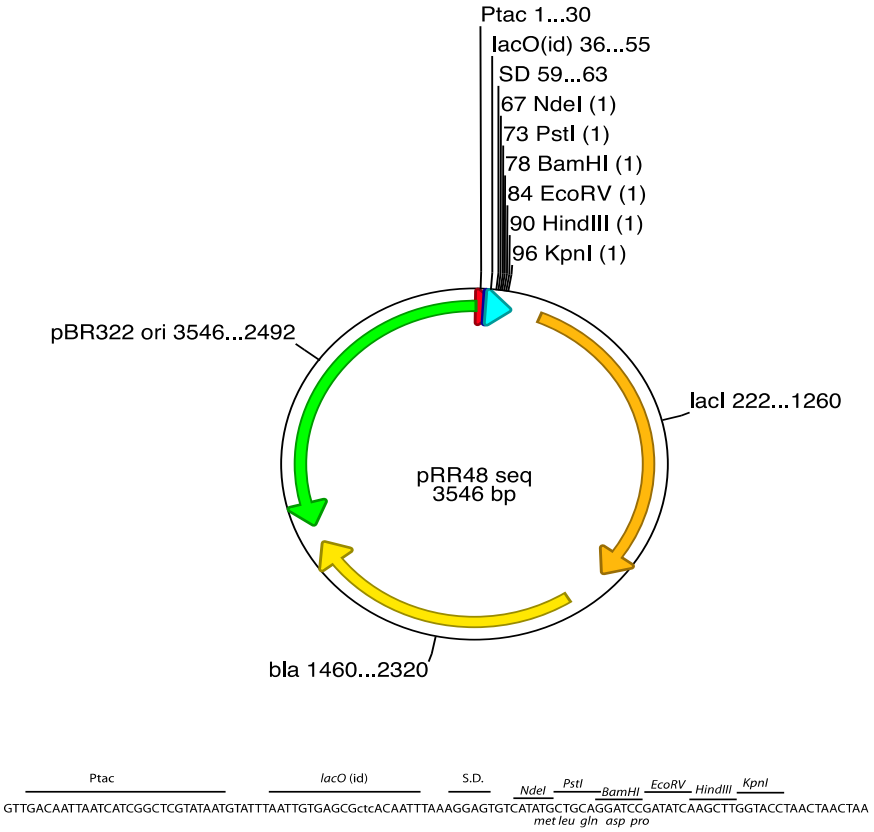


Figure 4. Plasmid map of pRR48. The parent plasmid, pRR48, is a medium copy number plasmid that is derived from pCJ30. it confers ampicillin resistance and expresses cloned genes from a Tac promoter and expression is regulated with IPTG.

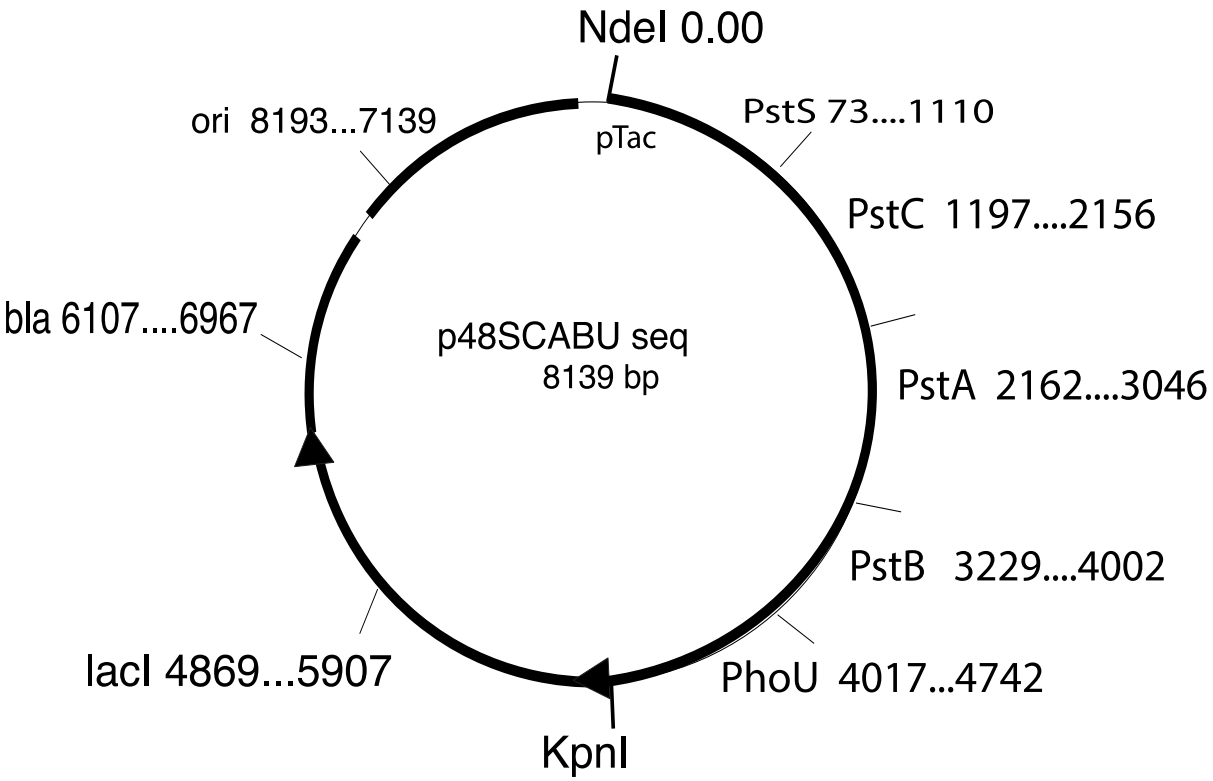


Figure 5. Plasmid map of p48SCABU. This is a plasmid map of the plasmid referred to in this thesis as p48SCABU. This is a plasmid that was constructed by inserting the *pst*SCABU genes into the pRR48 multiple cloning site between *Nde*I and *kpn*I sites.

2.3.5 Phosphate uptake measurements

Cells were grown overnight in 5 ml MOPS LoPi containing 0.1 mM IPTG after which they were washed twice with 5 ml MOPS medium free of glucose and phosphate. To completely starve the cells of phosphate, they were then re-suspended to an OD600 of ~0.45 in MOPS medium containing 0.4% glucose and 0.1 mM IPTG, but no phosphate. They were then incubated at 37°C on a roller drum for 2 h. Transport assays were performed at room temperature in which 750 µl of cells at an OD600 of 0.45 were added to 750 µl of a 10.5 µM solution of K_2HPO_4. After 45 seconds of incubation, 1000 µl of cells were removed and rapidly filtered through pre-wet 0.2 µm nitrocellulose filters using a Millipore 1125 vacuum filter apparatus. The filtrates were collected in glass tubes. Phosphate concentrations of each filtrate sample were then measured using the Malachite Green assay described above. Phosphate uptake was determined by subtracting the amount of phosphate (in nmoles) in the filtrate from the amount in a blank reaction containing no cells and dividing by the product of the dry weight of cells in each sample and the time. The dry weight of cells was estimated from the following conversion factors: OD600 of 1 = 1.11 X 109 cells/ml and each cell has a dry weight of 2.8 X 10-13 g. Each strain was assayed using two biological replicates in duplicate.

2.3.6 Transformations

Overnight cell cultures (3ml) in LB growth media with appropriate antibiotics were used as competent cells for transformations. First, the overnight cultures were diluted 1:100 into 25ml of fresh LB growth media and allowed to grow on a shaker at 37°C until the culture reached an OD_{600} between 0.3-0.4. These cells were then centrifuged for 10 minutes at 4 °C at high speed. The supernatant free cell pellet was re-suspended in 5 ml of cold 0.1 M Calcium chloride

solution followed by 30 min incubation on ice. After incubation on ice, cells were centrifuged and the pellet was re-suspended in 1ml of 0.1 M Calcium chloride solution.

To a clean micro-centrifuge tube, 100 ml of competent cells and 1-4 µl of plasmid DNA were added aseptically to initiate transformation. Another tube without DNA but only cells was used as a negative control. These tubes are then incubated on ice for 30 min followed by a 60-sec heat shock in a 42 °C water bath. Tubes were immediately transferred to an ice bucket for a 2-min incubation. 1 ml of LB media was added to these tubes and incubated at 37 °C for one hour. Finally, these cells were spread onto agar plates with proper antibiotics to select for transformed cells.

2.4 Results

This experimental plan tests the hypothesis that the PstB component of the PstSCAB protein transmits a phosphate sufficiency signal to the interior of the cell through a conformational change that is part of the catalytic cycle. Research has shown that the *pstSCAB* and *phoU* genes are necessary for phosphate signaling. To determine whether these genes could be complemented with plasmids, we expressed these two genes on a single plasmid (*pstSCABU*) in *E.coli* strain BW26337 (a Δ*pstSCAB-phoU* strain). Alkaline Phosphatase (AP) expression was used to analyze the Pho regulon expression. Fig. 6 shows AP assay of BW25113 (wild type), BW26337 with pRR48 (empty vector) and BW26337 with *pstSCABU* in MOPS low Pi media (black) and MOPS high Pi media (white). In the wild type cells (positive control), Pho regulon was expressed only under low Pi conditions and in the cells with an empty vector (pRR48, a negative control) high AP levels were observed both under high Pi and low Pi

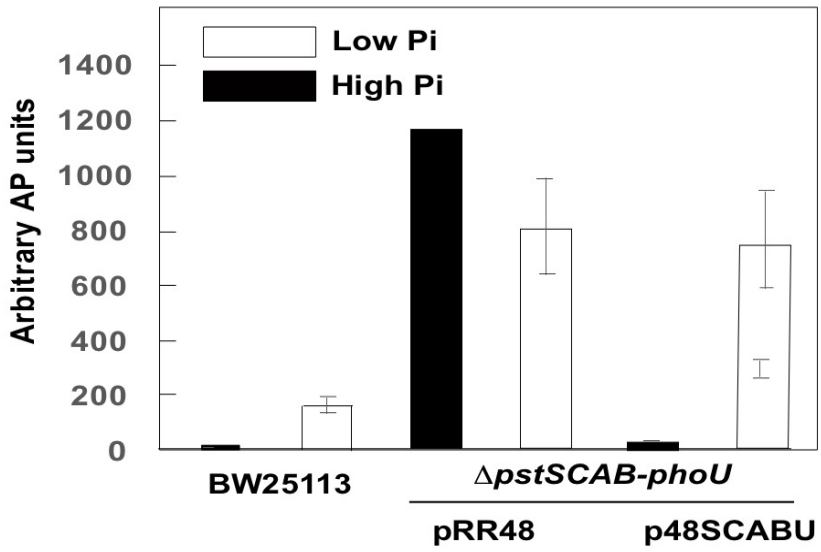


Figure 6. Complementation of Δ*pstSCAB-phoU* mutation with plasmids. Cultures of BW25113 (a wild-type strain), BW26337 (a Δ*pstSCAB-phoU* strain) cells with pRR48 (Empty vector), p48SCABU (*pstSCAB phoU*) were grown overnight in MOPS low Pi and MOPS high Pi media. Bacterial Alkaline Phosphatase activities were assayed in technical duplicates. Error bars represent the ± standard deviation.

conditions. When PstSCAB and PhoU proteins were expressed through plasmids, the AP expression pattern mirrored the wild type cells, suggesting that we can reproduce the signals through plasmids. Here, the magnitude of AP induction was lower in the complemented strain probably because of copy number effects from the plasmid vector.

In order to test our conformational signal model of the PstSCAB transporter, we first wanted to lock transporter in locked in open or closed conformations. (see Fig. 3). Several recent studies have shown that mutations exist at the conserved sites of proteins that lock transporters in either inward or outward conformations (MalEFGK2) (40,41). Previous genetic analysis of the *pstSCAB* genes have identified mutants that have phenotypes that are consistent with a nonfunctional transporter that is "locked" into a state which signals PhoR to be in its phosphatase state. Based on these ideas, we compared the PstB with MalK by using BLAST alignment. As can be seen in Fig. 7, Q160 and E179 of PstB correspond to Q140 and E159 in MalK. Therefore, we introduced mutations into *pstB* at 160th and 179th positons that are in the same positions as the *mal*K mutations that lock that protein into alternate conformations. As can be seen in the Fig. 7, the highest levels of conservation between the two proteins are found in the Walker A, Walker B, and ABC Signature motifs for ATP binding and hydrolysis. Fig. 8 presents a three-dimensional model of the PstB structure, created using a threading technique on the Phyre2 web site (47), which shows the positions of Q160 and E179. In the fully assembled transporter, these residues would lie at the dimer interface of PstB. The Q160K mutation is predicted to prevent binding of ATP to the transporter, locking the transporter in the inward-facing state whereas the E179Q mutation is predicted to prevent the hydrolysis of ATP, hence the transporter can be locked with ATP present, outward facing state.

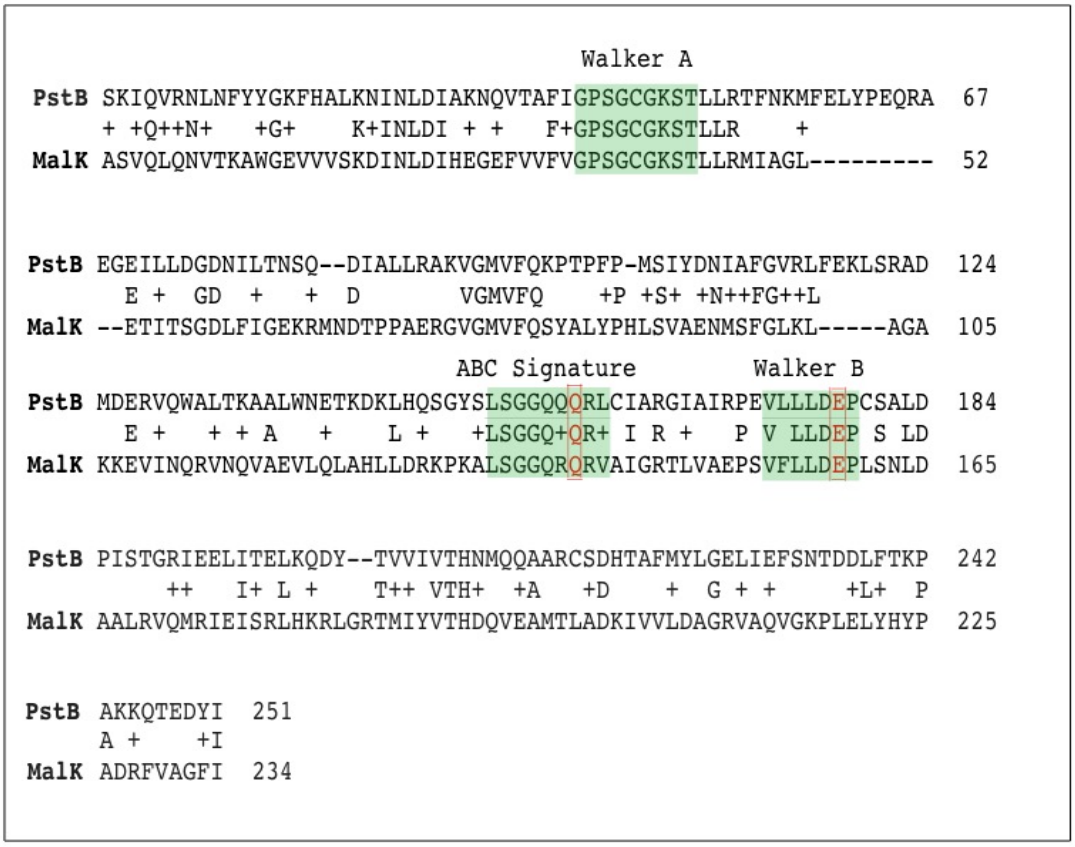


Figure. 7. Sequence alignment of PstB and MalK performed by European Molecular Biology Open Software Suite (EMBOSS). The protein sequences of PstB and MalK were aligned by BLAST to represent the three key conserved motifs (green). The red boxes in the ABC signature motif and Walker B motif shows the residues that were considered for the Q160K (a neutrally-charged Glutamic acid to posively-charged Lysine) and E179Q (a negatively-charged Glutamine to a neutrally-charged Glutamic acid) mutations on PstB, respectively.

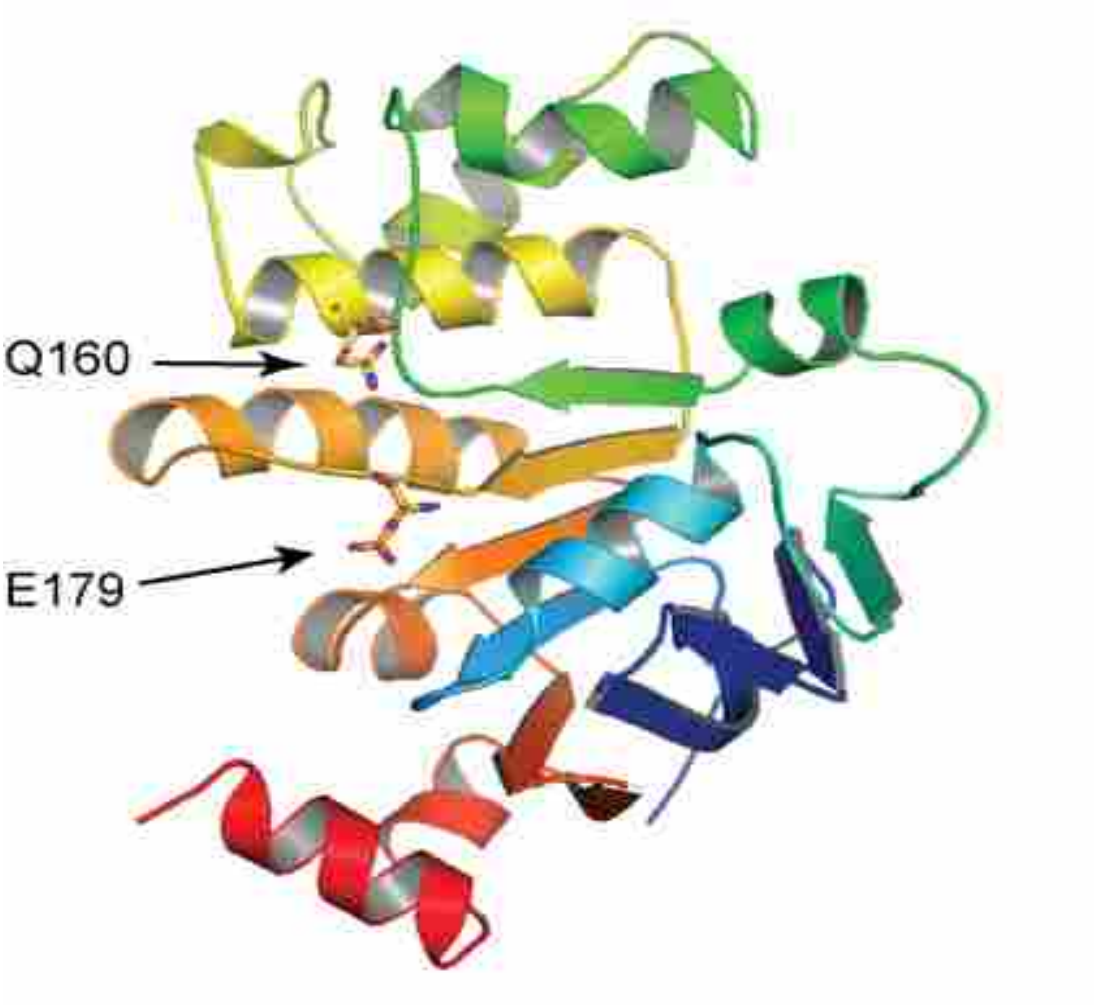


Figure. 8. Model of PstB created using the SWISS-MODEL website. The two arrows showing the PstB residues, Q160 and E179, that were chosen to mutate to lock the Pi transporter in the PstB open and the PstB closed conformations, respectively

We tested the Q160K and E179Q *pstB* mutants by introducing the plasmids expressing these genes into BW26337 (a Δ*pst*SCABphoU strain) and we looked at the signaling capabilities of these mutants by Alkaline Phosphatase (AP) Assays following seven hours of growth (Fig.9). As can be seen in Fig.9, high AP production was observed in the E179Q mutant of PstB both in the high-phosphate medium and in the low-phosphate medium. These results for E179Q mutant can be compared to the results of the deletion strain containing empty vector (high AP production). This observation is consistent with our model in that signaling a low-phosphate environment involves stabilizing an active kinase conformation in PhoR. Activated PhoR is also the phenotype of a Δ*pstSCABphoU* mutant. The results of *pstB*Q160K mutation are also consistent with our hypothesis. As seen in Fig. 9, the *pstB*Q160K mutation induced low

AP levels irrespective of the media concentrations. These results suggest that Q160K mutant are consistent with a signaling state that maintains PhoR in its "phosphatase-on/kinase-off" conformation, which corresponds to growth in a high-phosphate environment. To further strengthen our conformational signaling hypothesis, we performed phosphate transport assays for all our strains. The aim of this experiment is to understand whether the conformational changes in the PstSCAB transporter or the Pi transport through Pst transporter is the driving factor for signal transduction. Cells were grown in MOPS LoPi medium overnight and then incubated in phosphate-free medium to exhaust the cells of intracellular phosphate stores. Cells were then incubated in media containing 5.25 μM phosphate. Instead of measuring phosphate uptake directly using ^{32}Pi, we chose to measure phosphate depletion from the media using a nonradioactive method. As can be seen in Fig.10, we compared the phosphate transport of the PstB mutants (Q160K and E179Q) to cells carrying the wild type gene and to cells carrying an empty vector. The strain containing the wild type gene showed a 3-fold increase of phosphate

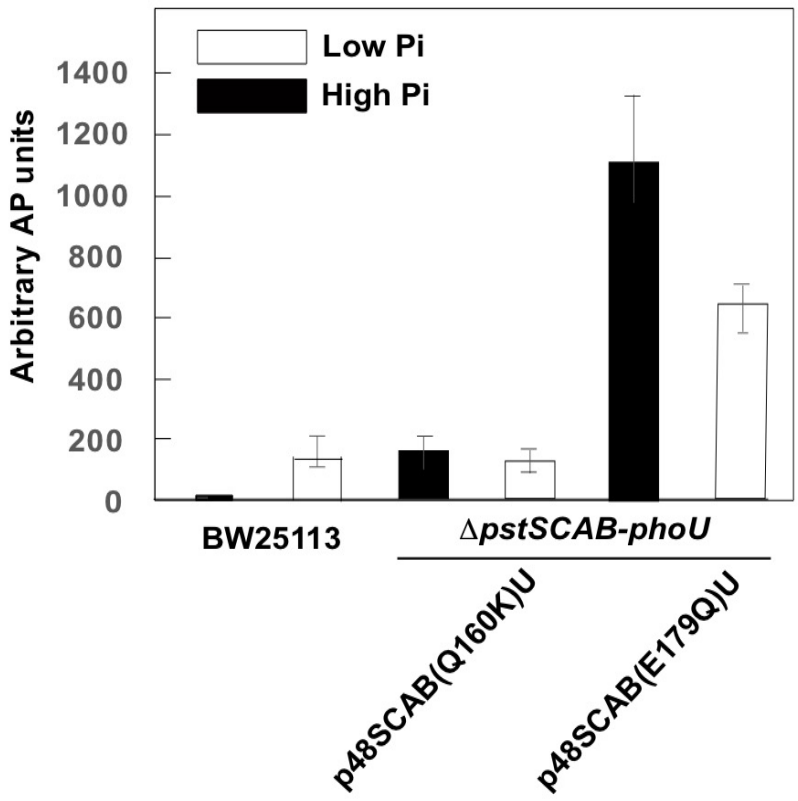


Figure.9. Phosphate-signaling in wild type cells and experimental strains. Cells were grown overnight in either MOPS LoPi or MOPS HiPi media, diluted in the morning and grown for an additional seven hours in the indicated media. Bacterial AP activities were performed in technical duplicates. Error bars represent ± standard deviation. BW25113 (wild type) and BW26337 cells harboring either p48SCAB(Q160K)U or p48SCAB(E179Q)U.

uptake when compared to cells that have an empty vector. Moreover, both the mutants show phosphate transport similar to that of the empty vector strain. The background levels of phosphate transport are most likely due to transport through the PitA and/or PitB secondary transporters of *E. coli.* These results show that the Pst transporters containing either the Q160K or the E179Q mutations are nonfunctional (no Pi uptake). As the transporter containing the PstB(Q160K) protein is still capable of signaling a phosphate-replete environment, even when phosphate is limiting, we conclude that signal transduction is independent of phosphate transport and likely results from the predicted conformational changes in the transporter.

In the course of our work, we observed some differences in phosphate-signaling, from experiment to experiment, especially when growth times were extended in low-phosphate media, and wanted to know if those differences were maintained at different time points throughout the growth curve. Therefore, we followed phosphate-signaling, as measured by AP expression, and measured media phosphate levels through the early stages of a growth curve for the wild type strain and the four experimental strains carrying plasmids. With this design, we were able to correlate phosphate-signaling to environmental phosphate levels in a single experiment. To perform the experiment, cells were grown in phosphate-replete media overnight and the following day, cells were washed in phosphate-free media, and then inoculated in defined media containing 60 μM phosphate, a concentration in which the Pho regulon is not inducted. At various time points thereafter OD_{600} readings were taken and cells and spent media were collected for AP analysis and for media phosphate concentrations, respectively. Fig. 11a shows that in the wild type cells, the AP expression turns on fully only when the environmental phosphate levels were below 5 μM (beginning from about 4.5 hours of the growth curve). Fig. 11b shows that in the Δ*pstSCAB-phoU* strain with an empty vector, the AP expression remains at

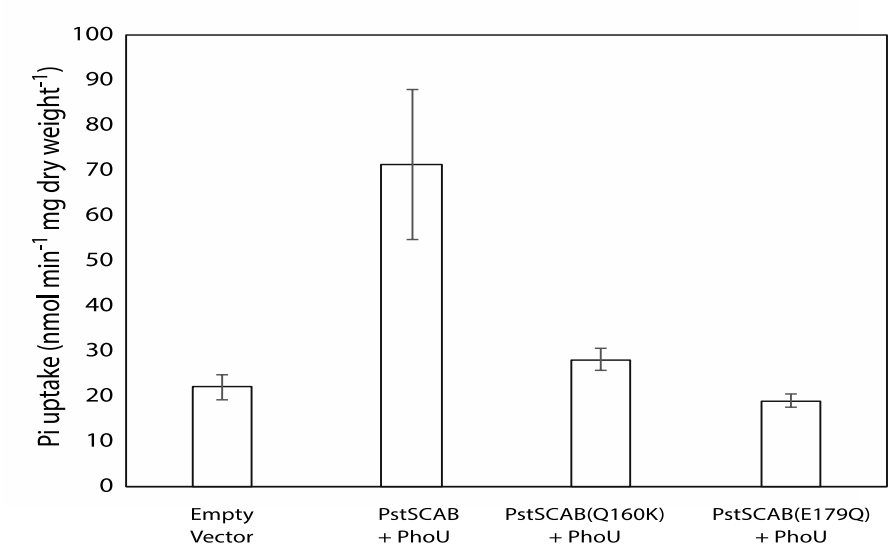


Figure.10. Phosphate uptake in the *pstB* mutant strains. Cultures of BW26337 cells with pRR48, p48SCABU, p48SCAB(Q160K)U or p48SCAB(E179Q)U were grown overnight in MOPS LoPi medium. After starving them of phosphate and then adding K_2HPO_4, phosphate uptake was measured. Each strain was assayed using two biological replicates in duplicate. Error bars represent ± the standard deviation.

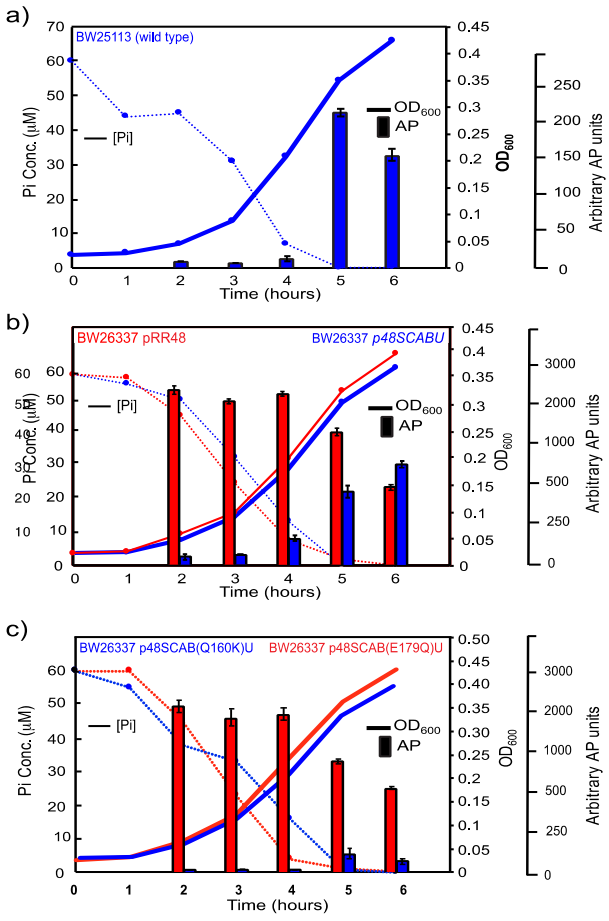


Figure 11. Induction of alkaline phosphatase expression upon phosphate starvation. Media phosphate concentrations are represented by dashed lines, cell growth as measured by OD600 readings are shown with solid lines, and Pi-signaling, as measured by AP expression, is shown with solid vertical bars. (a) Wild type strain, BW25113 (b) BW26337 strain carrying either pRR48 plasmid (Red) or p48SCABU (Blue) (c) BW26337 strain carrying either p48SCAB(Q160K)U (Blue) or p48SCAB(E179Q)U (Red)

a high level throughout this portion of the growth curve. The same strain carrying the p48SCABU plasmid showed low AP levels initially and induced AP expression when the phosphate levels fell below ~10 μM (beginning from 4 hours of growth curve). Fig. 11c shows that the PstB(Q160K) mutant always expressed low levels of AP, whereas the PstB(E179Q) mutant always expressed high levels of AP. We also noted a general decline in AP levels as the growth curve extended that were probably due to diminished synthetic potential. These results demonstrate that a constant phosphate-signal is transmitted by *pstB* mutants that are predicted to adopt stable conformations, even when environmental phosphate concentrations transition through a concentration gradient that normally activates the wild-type protein.

2.5 Discussion

The Pho regulon has been studied for many decades, yet is has remained unknown the molecular mechanisms by which *E. coli* cells recognize environmental phosphate. Earlier work has shown that the signal for the activation of the Pho regulon is not a change in the intracellular concentration of phosphate or the motions of the PstSCAB transporter (51). We have presented evidence to support a mechanism that involves the sensing of the alternate conformational states of the transporter. Though previous research identified the PhoBR two-component system as central in controlling the Pho-regulon, it remained unclear how PhoR sensed the phosphate levels. This study presented data that supports a model involving the sensing of alternate PstSCAB conformations. These PstSCAB conformations would normally be formed as part of the phosphate transport cycle, but in the present research, those conformations were achieved through mutagenesis of the *pstB* gene.

We were not able to providing biochemical data to further strengthen this hypothesis because previous work with PstSCAB protein has shown failure in overexpressing this protein. Moreover, introducing His-tags on either the N- or the C-terminus of the PstB altered signal transduction activity (Kristi Johns Master's Thesis, McCleary Lab). However, equivalent mutations to those we employed here for PstB have been used in numerous structural and biochemical studies on the other ABC transporters. For example, when MalK Q140K (equivalent to PstB Q160K) was incorporated into a transport complex with MalF and MalG, it showed very low ATPase activity and was completely defective in maltose transport (44). It was suggested that this variant of MalK locked the full transporter into an open, ground state. In separate studies on GlcV and MJ0796, which are isolated NBDs from the glucose transporter *and Sulfolobus sulfataricus* and a transporter of unknown cargo from *Methanococcus jannashii*, the conserved glutamate residues in the Walker B box were changed to glutamines (equivalent to the E179Q mutation in PstB) (52,53). In both cases, these mutations were shown to stabilize NBD dimerization, which corresponds to the closed conformation of PstB. A similar Glu to Gln mutation was introduced into the MsbA lipid flippase from Salmonella typhimurium, which was then reconstituted into nanodiscs (54). When examined by luminescence resonance energy transfer, this full transporter was also shown to be in closed, outward-facing conformation. While there is great diversity in the many ABC transporters found in nature, it is reasonable to propose that the mutations that were introduced into *pst*B may indeed have the proposed effects on the conformation of the PstSCAB transporter.

Recent work has demonstrated physical interactions between PstB, PhoU, and PhoR (32,33). It has been suggested that a complex of these proteins functions together to transmit information about environmental phosphate levels to the transcriptional machinery through

PhoB. We propose that PhoR senses the conformational states of the PstSCAB transporter within this complex to control its opposing kinase and phosphatase activities (see Fig. 3). The inward-facing, open conformation of the transporter would therefore interact with PhoR to promote its phosphatase activity. The outward-facing closed conformation of the transporter would be predicted to interact with PhoR to promote its kinase activity. Based upon these results from Gardner et al., it seems most likely that the direct protein interactions occur between the PAS domain of PhoR and PhoU and PstB (33).

Previous work by Hoffer and Tommassen, showed that PstS was not required for phosphate-signaling when the alternate phosphate transporters PitA or PitB were overexpressed (49). We believe that this observation suggests that there may be a low-affinity phosphate-binding site within the PstB protein that is exposed to its cytoplasmic surface that binds phosphate and that when bound, would stabilize the open, inward-facing conformation, but that under normal intracellular phosphate concentrations, the PstCAB transporter would exist in the closed, outward-facing conformation.

Chapter 3 The Phosphate-Binding Protein (PstS) of *Escherichia coli* Is Essential for P_i-Regulated Expression of the *pho* Regulon

3.1 Introduction

Generally, disruption of PstS leads to the constitutive expression of the Pho regulon. Previous work by Hoffer and Tommassen showed that PstS may not be required for phosphate-signaling when the alternate phosphate transporters PitA or PitB were overexpressed (49). Here, they studied the role of PstS in phosphate regulation of the Pho regulon by working with *pstA* or *pstC* mutants. They oberved that a strain with a *pstS* mutant that lacks phosphate transport system lost its proper expression control and this lost expression control was restored when PitA or PitB were overexpressed. They have reported that both pseudorevertants in *pit*A and wild-type *pit*A transporters can substitute for PstS in phosphate regulation. They also observed that like PitA activity, PitB activity can compensate for the absence of pstS. These researchers observed these results first in the K10 strain and later verified these same results in MG1655 and W3110 strains. Thus, in different genetic backgrounds, they were able to demonstrate that PitB activity can compensate for the absence of PstS. However, their results demonstrated that PstCABU is still required for Pho regulon expression even when PitB is active. These results challenge our view that extracellular rather than intracellular phosphate concentrations control the expression of the Pho regulon.

This idea is in apparent conflict with our hypothesis about P_i -signaling because PstS, being the phosphate binding protein and part of the transporter is absolutely necessary for signaling. Therefore, we wanted to replicate these experiments to find further clues in the

phosphate signaling mechanism. We overexpressed the PitA protein in the BW26337 (a Δ*pstSCABphoU* strain) and its variants and reported the results of signaling mechanism.

3.2 Materials and methods

3.2.1 Media

Cultures were grown in MOPS (morpholinepropanesulfonic acid) low phosphate and MOPS high phosphate minimal media (46). Low phosphate MOPS medium was prepared with 0.04% glucose (20% stock solution) and 0.1 mM phosphate (132mM K_2HPO_4 stock solution) and 1 X concentration of MOPS mixture. High phosphate MOPS medium was prepared with 0.06% glucose (20% stock solution) and 2 mM phosphate (132mM K_2HPO_4 stock solution) and 1 X concentration of MOPS mixture. The antibiotics ampicillin and chloramphenicol were used at 50 μg/ml and 40 μg/ml respectively when needed. All the MOPS media were sterilized by filtration. LB agar plates were used for culturing strains (1 liter LB broth with 14 grams of agar and ampicillin when needed).

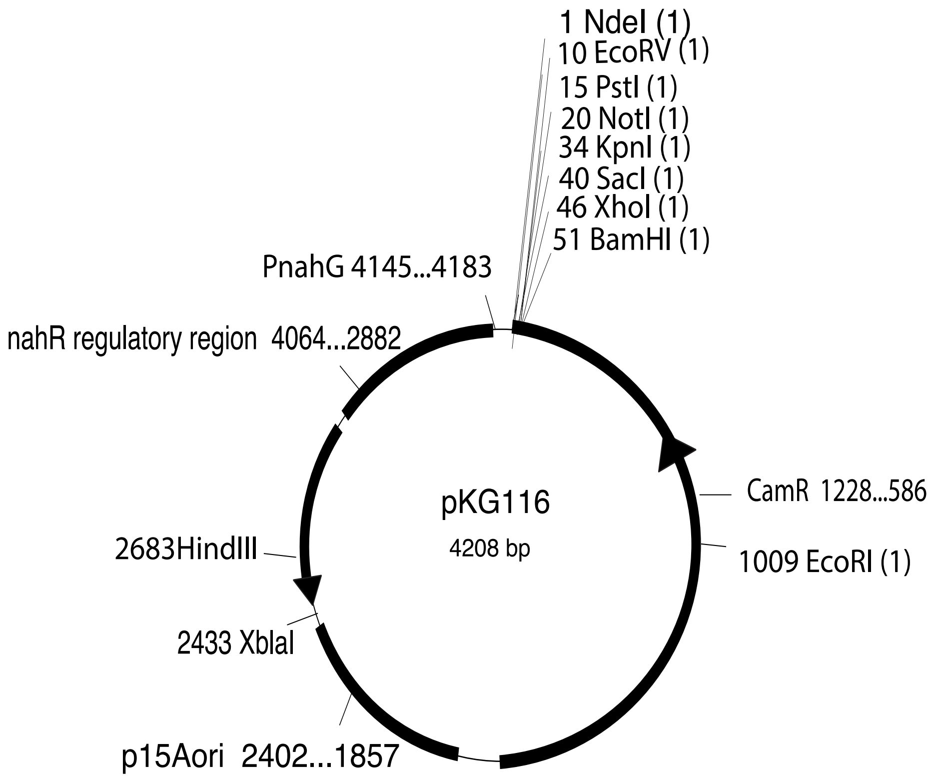
3.2.2 Strains, Plasmids and Growth conditions

E. coli BW25113 was used as the wild-type strain (50). Strain BW26337 contains a ΔpstSCAB-phoU::FRT mutation (50). The parent plasmids used in this study are pRR48 and pKG116 (45). pRR48 is a medium copy number plasmid that confers ampicillin resistance and expresses cloned genes from a Tac promoter (see Fig 4). pKG116 is a chloramphenicol resistant plasmid and expresses cloned genes can be controlled with sodium salicylate (see Fig 12). p48SCABU and p116CABU plasmids were constructed by amplifying the pstSCAB-phoU operon by PCR using primers that contained embedded NdeI and KpnI restriction sites (see Fig. 6 and Fig. 13). Following PCR, amplified DNA was cleaned with Quick-Change purification kit and the PCR product was digested with NdeI and KpnI restriction enzymes and ligated into a

similarly digested pRR48 and pKG116 plasmid vectors. The plasmids encoding mutant versions of *pst*B were p48SCAB(Q160K)U, p48SCAB(E179Q)U, p116CAB(Q160K)U and p48CAB(E179Q)U (see Table 1 for description). They are derived from p48SCABU and p116CABU and were constructed using the Quick-Change site-directed mutagenesis kit from Agilent Technologies. Here, the plasmids, p48SCABU and p116CABU, were amplified through PCR using two sets of forward and reverse primers that were designed to create point mutations to the 160th amino acid and 179th amino acid of PstB (see Table 2). These mutations were done separately. The mutant sequences were verified by DNA sequence analysis at the BYU DNA Sequencing Center. Strains were grown at 37°C with shaking.

3.2.3 Alkaline phosphatase assays

Cultures were grown overnight in MOPS LoPi medium at 37°C with shaking. 20 µl of overnight cultures were used to inoculate 2 ml MOPS HiPi or MOPS LoPi media and allowed to grow on a roller drum for 7 hours at 37°C. 600µl of these cultures were pelleted and then re-suspended in 600 µl of 1 M Tris-HCl pH 8.2. 100 µl of the cell suspension were then added to 100 µl of 1 M Tris-HCl pH 8.2 for a 1:2 dilution and the OD600 values were determined with a Thermo Scientific Multiscan FC 96 well plate reader. To the remaining 500 µl of cells, 10 µl of 0.1% sodium dodecyl sulfate and 20 µl of chloroform were added and the tubes were vortexed twice for 5 seconds at 20 second intervals. 50 µl of each sample were loaded into a 96 well plate containing 150 µl of 1 M Tris-HCl pH 8.2 and incubated at 37°C for 10 minutes to equilibrate the temperature. Following the incubation, 40 µl of 20 mM p-nitrophenyl phosphate in 1 M Tris-HCl pH 8.2 were added and OD420 values were determined at 1 minute intervals for 20 minutes



This expression system is inducible with submicromolar concentrations of sodium salicylate.

```
       -35?                    -10?                                        S.D.    NdeI     EcoRV     NotI          KpnI
     1 TTTATCAATATTGTTTGCTCCGTTATCGTTATTAACAAGTCATCAATAAtGCCATaAgGAGTACCATATGcatgatatctgcagcggccgcactagTGGT 100
PnahG  >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>                             M  H  D  I  C  S  G  R  T  S  G

           SacI      BamHI
   101 ACC GAGCTCCTCGAGGATCCGCGGATAAA 129
```

Figure 12. Plasmid map of pKG116. The parent plasmid, pKG116, is a medium copy number plasmid that is derived from pLC112. it confers chloramphenicol resistance and contains the positive regulatory gene, nahR, and the nahG promoter upstream of multiple cloning site and from which expression is controlled with sodium salicylate.

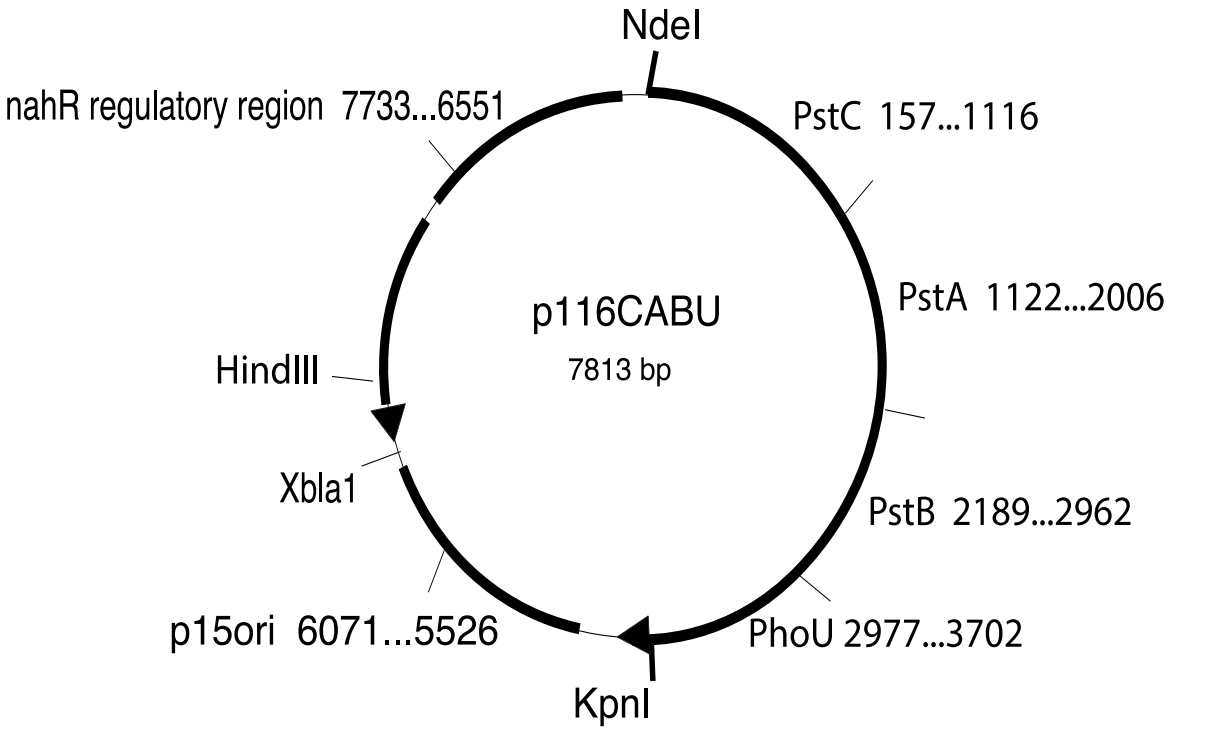


Figure 13. Plasmid map of p116CABU. This is a plasmid map of the plasmid referred to in this thesis as p116CABU. This is a plasmid that was constructed by inserting the pstCABU genes into the pKG116 multiple cloning site between NdeI and kpnI sites.

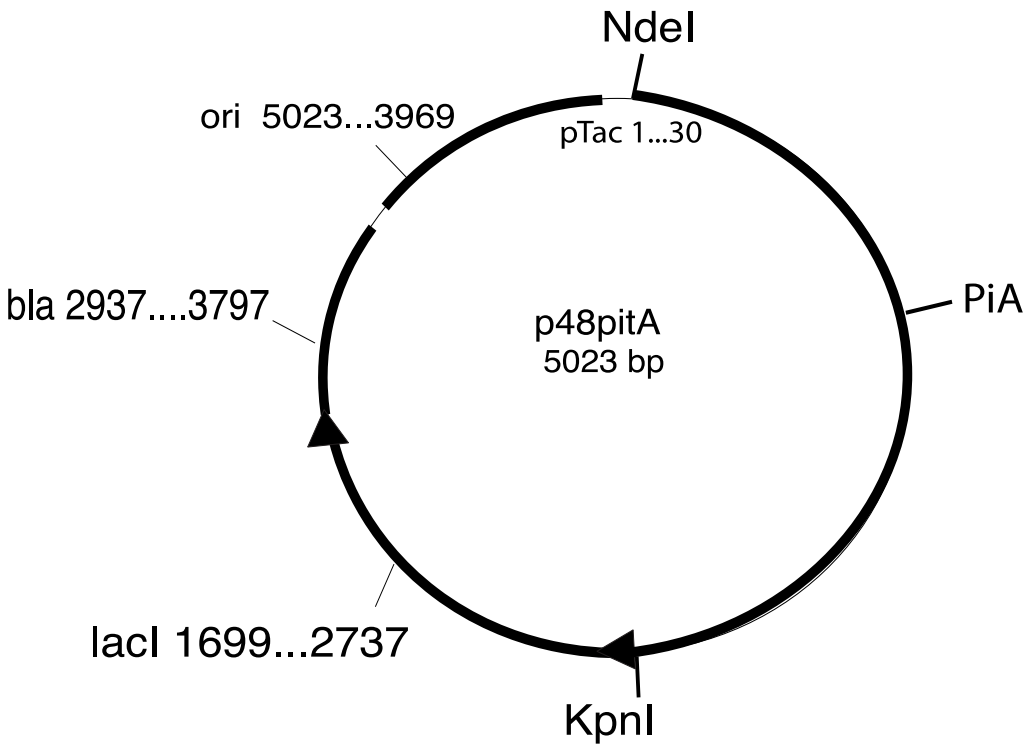


Figure 14. Plasmid map of p48PitA. This is a plasmid map of the plasmid referred to in this thesis as p48PitA. This is a plasmid that was constructed by inserting the *pitA* genes into the pRR48 multiple cloning site between *Nde*I and *kpn*I sites.

and the maximum kinetic rates for each sample were measured (ΔA420/min). Finally, arbitrary AP units were calculated as (1000 × maximum kinetic rate)/(2 × OD600 of the overnight culture). Each strain was assayed using two biological replicates in duplicate. The average AP values of each sample with error bars representing standard deviations were reported.

3.2.4 Transformations

Overnight cell cultures (3ml) in LB growth media with appropriate antibiotics were used as competent cells for transformations. First, the overnight cultures were diluted 1:100 into 25ml of fresh LB growth media and allowed to grow on a shaker at 37°C until the culture reached an OD_{600} between 0.3-0.4. These cells were then centrifuged for 10 minutes at 4 °C at high speed. The supernatant free cell pellet was re-suspended in 5 ml of cold 0.1 M Calcium chloride solution followed by 30 min incubation on ice. After incubation on ice, cells were centrifuged and the pellet was re-suspended in 1ml of 0.1 M Calcium chloride solution.

To a clean micro-centrifuge tube, 100 ml of competent cells and 1-4 µl of plasmid DNA were added aseptically to initiate transformation. Another tube without DNA but only cells was used as a negative control. These tubes are then incubated on ice for 30 min followed by a 60-sec heat shock in a 42 °C water bath. Tubes were immediately transferred to an ice bucket for a 2-min incubation. 1 ml of LB media was added to these tubes and incubated at 37 °C for one hour. Finally, these cells were spread onto agar plates with proper antibiotics to select for transformed cells.

3.3 Results and Discussion

This experimental plan tests the hypothesis that the overexpression of the low affinity secondary phosphate transporter, PitA, can substitute for the periplasmic phosphate binding

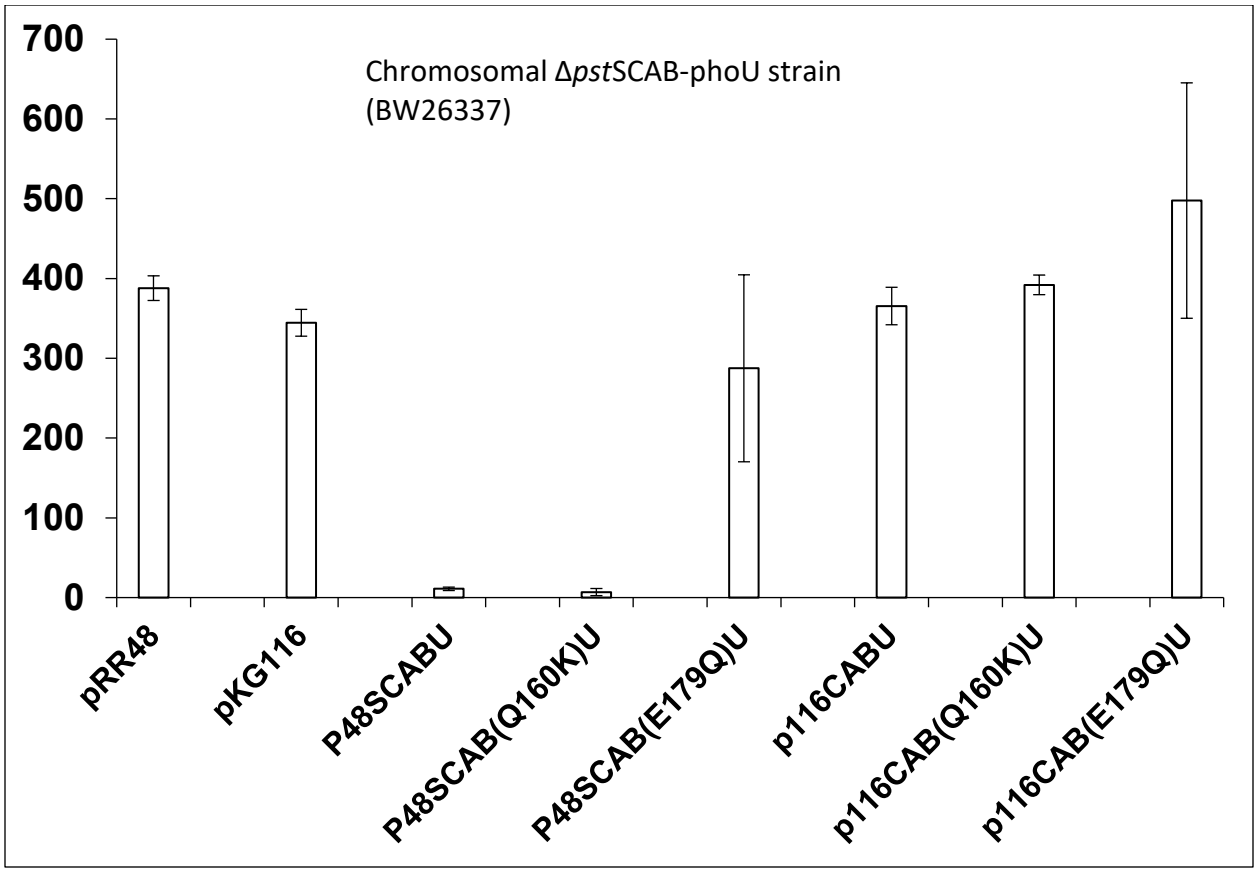


Figure 15. PstS is required for phosphate signaling. Duplicate cultures of the BW26337 strains expressing PstS (PstSCABU and its *pstB* mutants, represented by bars 3-5) and not expressing *pstS* (PstCABU and its *pstB* mutants, represented by bars 6-8) were grown in high P_i media and assayed for Bacterial Alkaline Phosphatase activity. pRR48 and pKG116 are the parent plasmids for PstSCABU and P116CABU constructs respectively and are included as empty vector controls. Error bars represent ± standard deviation.

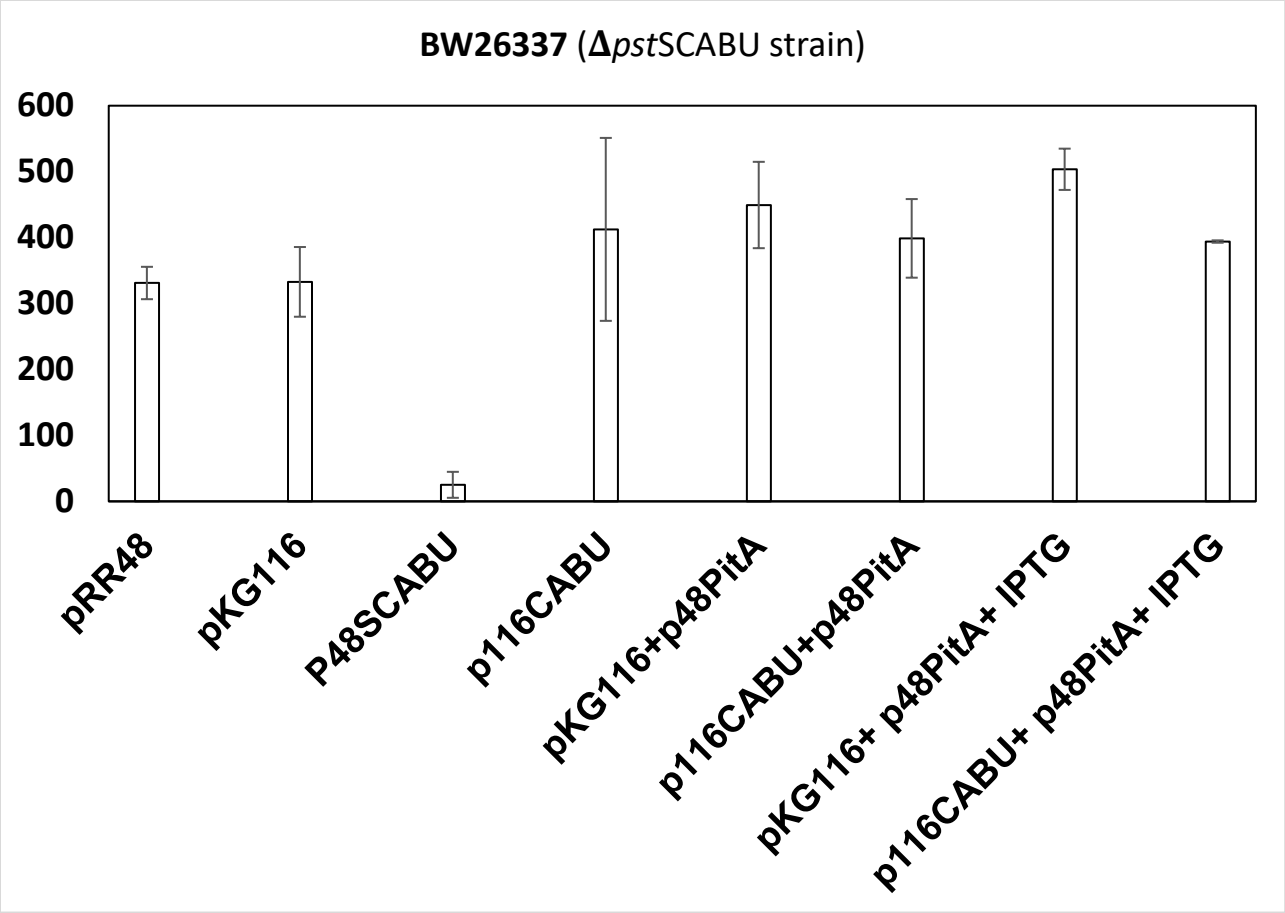


Figure 16. Overexpression of PitA in Δ*pstS* strains. Duplicate cultures of the BW26337 strain expressing PstS (PstSCABU), strain not expressing PstS (p116CABU), strains where PitA is expressed (represented by p48PitA plasmid) and strains where PitA is overexpressed (through IPTG) were grown in high P_i media and assayed for Bacterial Alkaline Phosphatase activity. pRR48 and pKG116 are the parent plasmids for PstSCABU and PstCABU constructs respectively and are included as empty vector controls. Error bars represent ± standard deviation.

protein, PstS. To test this hypothesis, we created Δ*pstS* strains. First, we cloned the *pst*CABU genes into pKG116 plasmid and introduced them into BW26337 (a Δ*pstSCABphoU* strain). We then tested the signaling capabilities of this mutant by AP assay. As can be seen in Fig. 15, when pRR48, pKG116 were introduced into this genetic background, the cells always expressed high AP in high phosphate medium (controls). When the whole *pst*SCABU operon was expressed from plasmid, very low AP production was observed. However, when we knocked out the *pstS* and only expressed *pstCABU*, the AP production was elevated almost 400-fold. Moreover, this same pattern of expression was also observed in our *pstB* mutants, p116CAB(Q160K)U and p116CAB(E179Q). This suggests that the periplasmic, P_i binding- PstS protein is necessary for the signaling of external P_i. Without it, the transporter is blind to external phosphate.

We then overexpressed PitA by cloning into the pRR48 plasmid (p48PitA, IPTG inducible, see Fig. 14) in Δ*pstS* mutants and observed the signaling pattern in these mutants. As can be in Fig 16, AP expression patterns remained the same in all the Δ*pstS* mutants (high AP levels). These results suggest that even when the alternate phosphate transporter, PitA, is overexpressed, PstS is still required for phosphate signaling and PitA overexpression cannot compensate for PstS loss.

Thus, our results were contradictory to the previous results of Hoffer and Tommassen. It is possible that we would have seen different results if we had overexpressed both PitA and PitB together in Δ*pstS* background. Because *pstCAB phoU* was required for Hoffer and Tommasens's results, we believe that this suggests that there may be a low-affinity phosphate binding site within the PstB protein that is exposed to its cytoplasmic surface that binds phosphate. This phosphate-bound low-affinity site would stabilize the open, inward-facing conformation but that

under normal intracellular phosphate concentrations, the PstSCAB transporter would exist in the PstB-closed, outward-facing conformation.

Chapter 4 Future Directions

During our growth curve experiments in chapter 2, we had problems measuring the AP expression at low cell densities within the growth curve. To overcome this problem, we have tried to find an alternative to the AP assay for analyzing the Pho regulon by expressing the green fluorescent protein (gfp) from the *pho*A promoter. However, gfp expression had affected the growth of many strains. Some of the future experiments in our lab might include analyzing many constructs and their growth curves. Therefore, finding an alternative to AP assay could be one future direction.

As mentioned in chapter 3, overexpression of PitA and PitB could restore signaling of Pho regulon in the mutant PstS strains. However, our results showed that PstS is required for signaling external phosphate. Future work involves confirming these results by western blotting analysis. Through this technique, we can demonstrate that the PstSCABU, PstCABU and PitA proteins are expressed. Performing phosphate transport assays with $^{32}P_i$ may allow us to compare phosphate uptake in our experimental strains which might help in understanding phosphate signaling mechanism. Future work looking at the signaling pattern when both the secondary transporters (PitA and PitB) are overexpressed in the Δ*pstS* background can help us understand the roles of PitA and PitB in external phosphate sensing and its transport mechanism.

Table 1 Strains and Plasmids

Strain or plasmid	Description	Reference
E.coli strains		
BW25113	Wild type	17
BW26337	BW25113 Δ*pstSCAB-phoU::FRT*	17
BW26337 p48SCAB(Q160K)U	*pstSCABU plasmid*, with *a mutation on PstB (Q160K)*	This study
BW26337 p48SCAB(E179Q)U	*pstSCABU* plasmid, with *a mutation on PstB (E179Q)*	This study
BW26337 p116CABU	BW25113 Δ*pstS*	This study
BW26337 p116CAB(Q160K)U	BW25113 Δ*pstS with a mutation on PstB (Q160K)*	This study
BW26337 p116CAB(E179Q)U	BW25113 Δ*pstS with a mutation on PstB (E179Q)*	This study
Plasmids		
pKG116	pACYC184-based replicon, Cam^r, nahR	18
pRR48	pBR322-based replicon, Amp^r, $lacI^q$	20
p48SCABU	*pstSCABU* expression plasmids, IPTG inducible	This study
p48SCAB(Q160K)U	*pstSCABU with a mutation on PstB (Q160K)*	This study
p48SCAB(E179Q) U	*pstSCABU with a mutation on PstB (E179Q)*	This study
p116CABU	*pst*CABU expression plasmid	This study
p116CAB(Q160K)U	*pst*CABU *with a mutation on PstB (Q160K)*	This study
p116CAB(E179Q)U	*pst*CABU *with a mutation on PstB (E179Q)*	This study
p48PitA	*pit*A expression plamid, IPTG inducible	This study

Table 2 List of primers used in this study

Primer name	Sequence in 5' to 3' direction
Q160K forward	TCTCTCTCTGGTGGTCAGCAAAAGCGTCTGTGT
Q160K reverse	ACACAGACGCTTTTGCTGACCACCAGAGAGAGA
E179Q forward	GCTGCTGCTCGACCAGCCGTGTTCGGCGC
E179Q reverse	GCGCCGAACACGGCTGGTCGAGCAGCAGC
PstC forward	CTGCAACCAAGCCTGCTTTT
PhoU reverse	GTCGGTACCTTATTTGTCGCTA

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