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Genetic analysis of conserved residues in PhoU of *Escherichia coli*

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GENETIC ANALYSIS OF CONSERVED RESIDUES IN P_{HO}U
OF *ESCHERICHIA COLI*

by

Stewart G. Gardner

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the degree of

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BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

GENETIC ANALYSIS OF CONSERVED RESIDUES IN PHOU OF *ESCHERICHIA COLI*

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Master of Science

The Pho regulon is controlled by the PstSCAB transporter, PhoU, and the two-component proteins, PhoB and PhoR. PhoU is a negative regulator of the Pho regulon under phosphate-replete conditions. How PhoU functions is unknown. Many PhoU homologues are found widely throughout prokaryotic domains. There are several conserved amino acid residues in the PhoU protein. It is hypothesized that these residues play an important role in the function of PhoU. To test this hypothesis, several site directed mutations in the *phoU* gene have been produced with single amino acid changes in conserved residues. After testing these mutants, it was found that some of the mutants abolished repression of the Pho regulon while other mutants had little or no effect. Further study of these mutants and their phenotypes will reveal more about how PhoU functions and help to better understand bacterial signaling in general.

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Introduction

Bacteria need to adapt to their environment in order to survive and thrive in multiple niches. This adaptation is often mediated through a two-component signal transduction system (14). There are many of these systems in bacteria that sense a signal and, through a signal cascade, adjust gene expression to adapt to the changing environment. One of these two-component systems in *Escherichia coli* helps to deal with phosphate limitation through expression control of the Pho regulon. Pho regulon expression is controlled by the PstSCAB transporter, PhoU, and the two-component proteins, PhoB and PhoR (Figure 1). Alkaline phosphatase (AP) is a protein under Pho regulon control and is often used to test for Pho regulon expression. This expression control is essential for the bacteria to survive and thrive in different environments.

The Pho regulon contains at least 31 genes that are under common expression control (33). In studies of global gene regulation by two-dimensional gel electrophoresis during phosphate starvation of cells, up to 208 proteins showed induction and 205 proteins showed repression (29). Clearly, the cell's ability to adapt to changes in environmental phosphate is important.

The control of Pho regulon genes is mediated through the action of various proteins. PhoR, a histidine kinase, can activate or repress expression. It acts through autophosphorylation that in turn activates PhoB, the response regulator. Phospho-PhoB then associates with specific DNA sequences in various promoters, called Pho boxes, to activate gene expression. PhoR also contains phosphatase activity to repress the system in phosphate-replete conditions. Other important proteins in this regulation include the phosphate specific transporter (PstSCAB) complex and PhoU. The Pst proteins form an

ATP binding cassette (ABC) transporter that is specific for the transport of inorganic phosphate (P_i) across the membrane. This transporter is also important for somehow signaling phosphate sufficiency through PhoU to PhoR by a process not yet understood. There are two main theories on how this PhoU mediated signaling occurs. One is that PhoU interacts with either the Pst transporter, PhoR, or both to pass along the signal. The other theory is that PhoU may act as an enzyme whose substrate or product is an intermediate in this signaling pathway. This regulation is important for cell growth and may also play a role in pathogenesis (7). This work looks at PhoU and its possible roles in phosphate regulation by studying mutations in highly conserved residues of PhoU. Better understanding of this system may lead to increased knowledge of bacterial signaling and other two-component signal transduction systems.

PhoR

The PhoR protein is the histidine kinase of the Pho regulon's two-component signal transduction system. It is a member of the class I family of histidine kinases, meaning its phosphorylation site is adjacent to the catalytic region (9). It receives a signal in low P_i environments which leads to autophosphorylation on a conserved histidine residue through an ATP-dependent mechanism. When phosphorylated, phospho-PhoR donates its phosphate to PhoB which leads to increased expression of the Pho regulon components. PhoR has also demonstrated a phosphatase activity, which allows it to inactivate phospho-PhoB in P_i -replete environments. This allows for tight control of the Pho regulon (9).

Histidine Kinases

There are certain activities and structures that are associated with histidine kinases in general. For activation, the autophosphorylation occurs between two homodimers. Each catalyzes the phosphorylation of the other's conserved histidine residue (26). Histidine kinase molecules also have distinct regulatory regions that work to mediate their response. Many have a periplasmic sensing domain that allows the molecule to directly receive a signal from the environment. They also have a kinase core. This core is composed of a dimerization domain and a catalytic domain that binds ATP and leads to phosphoryltransfer. The linker domain of these molecules is thought to act as a relay of conformational change to activate the kinase core on reception of a signal. The sensing domains of histidine kinases are less conserved. This may allow specificity to the individual signals each histidine kinase senses. Some histidine kinase molecules contain a Per-Arnt-Sim (PAS) domain. These domains sense different changes in the environment (redox potential, light, oxygen, or small molecules) depending on their cofactors (26).

Studies of PhoR

There have been many studies of the histidine kinase of the Pho regulon, PhoR. The *phoR* gene is located in an operon with *phoB*. To look at the specific function of PhoR in this system, one group constructed various *phoR* deletion mutants. Attempts to purify the full length protein were unsuccessful. So, plasmids that lacked the hydrophobic amino (N) terminal portion of the protein were cloned. From this plasmid, a soluble protein fraction that maintained function was isolated. Using this protein fraction, polyclonal antibodies against PhoR were produced. Then, using these anti-PhoR

antibodies, the cellular localization of the PhoR protein was observed. Full length PhoR protein was localized in the inner membrane fraction, but the truncated, soluble PhoR was found in the cytoplasm. Given these results, it has been concluded that the N terminal domain of PhoR acts as a membrane anchor and that the C terminal end maintains the enzymatic activity of the protein (37).

Another study that used PhoR mutations further elucidated the functions of this protein. By using precise deletions of PhoR, the phosphatase activity of PhoR on phospho-PhoB was confirmed. This activity was localized within the histidine phosphorylation domain of PhoR (5).

There have been difficulties in working with PhoR. As a membrane protein, it is difficult to isolate for purification, crystallization, etc. Also, clonal variations have been observed while working with mutants of PhoR (31, 32). It was found that several mutations led to constitutive AP expression or variable behavior. This makes it difficult to work with and interpret results because of the different phenotypes of the variants.

One important thing that is still unknown about PhoR is how it receives the signal of phosphate conditions. PhoR does not appear to have a receptor domain on the periplasmic portion of the protein that can directly sense phosphate or interact with ligands. It must receive its signal by some other means.

PhoB

The PhoB protein is a response regulator that, when phosphorylated, can activate the genes of the Pho regulon. PhoB receives its phosphate mainly from PhoR. However, PhoB can also be phosphorylated by acetyl phosphate and the CreC protein (33, 34).

First, some general details of response regulators will be discussed and then PhoB will be discussed in more detail.

Response Regulators

Response regulators catalyze the transfer of a phosphate from a conserved histidine on the histidine kinase to a conserved aspartate on its own regulatory domain (26). Other molecules can also donate their phosphates to different response regulators, acetyl phosphate, carbamoyl phosphate, etc. Response regulators generally consist of two domains. One domain is the N-terminal regulatory domain and the other is the C-terminal effector domain. Most response regulators mediate transcriptional control by binding to DNA with the C-terminal effector domain (26).

Studies of PhoB

There have been many studies on PhoB. There are studies that investigate the structure and domain functions of PhoB. Other studies compare PhoB to other response regulators. There is also a group of studies that look at the PhoR independent activation of PhoB and the possible cross-regulatory implications.

One study looked at PhoB's binding to DNA (4). The crystal structure of PhoB alone and PhoB bound to the *pho* box of the *phoA* operon was observed. It was found that PhoB binding to DNA has a novel tandem arrangement where monomers bind successively to 11bp repeats. It was also found that the transactivation loops of PhoB

appear in the proper orientation to interact with the σ^{70} subunit of RNA polymerase. This interaction may be the method by which PhoB can control gene expression (4).

Through analysis of mutants of σ^{70} , another group was able to confirm the regulatory interaction of PhoB and σ^{70} (17). It was seen that with certain mutations found near a helix-turn-helix motif in region 4.2 of σ^{70} there was a loss of the ability to express *pho* genes. These and other mutations were tested with an *in vitro* assay of transcription of the *pstS* promoter. It was found that these mutations inhibited expression whereas another, unrelated σ^{70} mutant allowed for constitutive expression while in the presence of PhoB. As a control, a *P_{tac}* promoter was used and it was found that expression was only marginally affected by the σ^{70} mutations (17). These results confirm that the method of gene control used by PhoB involves interacting with the σ^{70} subunit of RNA polymerase.

To further characterize this interaction, another study identified the DNA binding domain of PhoB (19). It was found that there are four amino acid positions in this DNA binding domain that are important for interactions with RNA polymerase. Based on the structure of the DNA binding domain of histone H5, it was observed that these positions are placed around a turn between two helices. There are three PhoB binding sites in the *pstS* promoter and it was found that while there is some intrinsic bending of the promoter, adding PhoB greatly enhances this bendability (19). This study further elucidates the mechanism by which PhoB activates the Pho regulon genes.

Using NMR, another group solved the structure of the DNA binding/transactivation domain of PhoB (23). It was found that this domain consisted of an N-terminal four-stranded β -sheet, three helices in a bundle, and a β -hairpin on the C-terminal end. There is a helix-turn-helix motif that is formed by the second and third

helices. It was found that the region where PhoB interacts with the RNA polymerase, a long turn region, differs entirely from the turn region of OmpR that interacts with the α subunit of RNA polymerase. The rest of the structure of PhoB was quite similar to that of OmpR. The PhoB/DNA interactions were also studied. It was found that two PhoB molecules bind within the Pho box. It was seen that the third helix recognizes the major groove TGTCA sequence, while the β -hairpin interacts with the minor groove non-specifically (23).

Another group was able to solve the structure of the receiver/dimerization domain of PhoB (24). It was found that the structure was similar to other α/β doubly wound folds found in other receiver domains of response regulators. With this crystal structure, it was predicted that a particularly hydrophobic region is the dimerization surface (24).

In a study of conserved acidic residues in the regulatory domain of PhoB, it was seen that some of the residues were essential for proper function while others lead to constitutive activity (41). An interesting result was that only some of the mutants were phosphorylated by acetyl phosphate and that most of the mutants had defects in their ability to bind magnesium, implicating magnesium as an important cofactor in PhoB function (41).

Looking at PhoB, NtrC, and CheB (all response regulators) fused with the DNA binding domain of λ repressor, another group saw an interesting trend (11). It was observed that the receiver modules of both NtrC and PhoB contained potential dimerization domains. From this result, NtrC and PhoB are classified as class I response regulators where activation is inhibited by interaction of the receiver and output domain, but this inhibition is relieved upon phosphorylation, inducing activation and dimerization

of the receiver modules. CheB is classified as a class II response regulator that functions in a similar manner, but does not dimerize upon activation (11).

Another study attempted to better understand the mechanism of control of PhoB (10). It was found that by comparing the DNA-binding domain of PhoB to a full length PhoB that the DNA-binding domain bound to the *pho* box with seven times greater affinity than an unphosphorylated PhoB. It was also found that this activation was greater than full length PhoB. From this data, it was concluded that the unphosphorylated receiver domain acts to silence the output domain of PhoB. Thus, when phosphorylated, the receiver domain relieves the inhibition on the DNA binding domain (10).

A study of different regions in PhoB added another dimension to the understanding of phosphorylation-mediated inhibition within PhoB (1). This study looked at proteins with deleted domains and at protein chimeras where they combined the N-terminal end of CheY (a response regulator involved in chemotaxis) with the output domain of PhoB. Through the various activities, it was found that the inhibition occurs between the $\alpha 5$ helix of the unphosphorylated receiver domain and the output domain (1).

Utilizing chimeras, another study tried to better understand PhoB as well as OmpR (30). OmpR is a response regulator whose N-terminal domain stimulates DNA binding when phosphorylated. To look at the interdomain linkers of these proteins, chimeras of various combinations of domains and linkers from PhoB and OmpR were constructed. It was seen that for the N-terminal domain of either protein to function as it normally does, it required its cognate interdomain linker. So, the N-terminal domain of OmpR could not activate upon phosphorylation without its interdomain linker and PhoB could not inhibit activation when un-phosphorylated without its interdomain linker (30).

One interesting topic involving PhoB that has been the focus of much research is its ability to be phosphorylated by not only PhoR, but by acetyl phosphate and CreC. This activity has been theorized to be a mechanism for cross regulation of the Pho regulon. CreC is in an operon of unknown function. The genes in this operon likely regulate an unknown set of target genes. CreC is a cytoplasmic membrane protein and appears to be a sensor molecule. Some speculate that CreC senses a catabolite or an extracellular ligand. It is possible that CreC may regulate its target genes through activating PhoB (34).

The possibility of cross regulation with acetyl phosphate is exciting because as a metabolite in a major phosphate utilization pathway, it may signal important information about the cell's current condition. However, with both CreC and acetyl phosphate, the effects of their phosphorylation of PhoB are only seen in the absence of PhoR. This raises the question of whether these mechanisms actually have a physiological impact on the signaling or are just too weak under proper regulatory conditions to cause a significant change in gene expression (34).

PstSCAB and other phosphate transporters

The phosphate specific transporter (Pst) system consists of the PstS, PstC, PstA, and PstB proteins. These form an ABC transporter that functions to transport P_i with high affinity into the cell. This system is similar to other transporters for histidine, ribose, maltose, as well as other periplasmic transport systems. PstS is the periplasmic P_i binding protein. It binds P_i with high affinity and has been predicted to be the primary sensor in this system of external P_i (16, 33). There are two integral membrane proteins that form a pore in the inner membrane, PstC and PstA. Finally, PstB acts to hydrolyze ATP to

power the transport. It is thought that PstB is essential in signaling because when the ABC motif is mutated, P_i uptake and repression are lost. However, it is also seen that P_i transport and signaling can be uncoupled by mutating PstA (6, 34).

There have been many studies of this P_i transport system, as well as others. A few older studies focused on the nucleotide sequence of the Pst system (28, 40). Five open reading frames were found in an operon that encoded for PstSCAB and PhoU, in that order.

Other phosphate transporters have also been found. PitA is a protein that transports metal phosphates. It is constitutively expressed and functions by utilizing a proton motive force to transport metal phosphates into the cell. Another P_i transporter, PitB, was found in a revertant strain that was a *pitA pstS* double mutant found to grow on P_i (15).

Another study further characterized PitA and PitB. It found that PitB is regulated under the Pho regulon (13). It was also found that when PitA or PitB expression is increased, they could mediate the P_i regulated expression of the Pho regulon in the absence of PstS (16).

These findings have implications in the mechanism of regulation. They imply that a signal is not solely transferred from the PstSCAB transporter to PhoU, but may also pass through PitA or PitB or that the signal is based solely on the level of phosphate sensed and is independent of the mode of P_i transport.

PhoU

As previously discussed, PhoU is part of the *pstSCAB-phoU* operon and acts as a negative regulator on the Pho regulon. PhoU is not essential for P_i transport but is required for repression of the Pho regulon (33). The mechanism of this negative regulation of the phosphate response is not known. PhoU has also been seen to play a role in phosphate chemotaxis in *Pseudomonas aeruginosa* (35). Initially, *phoU* was identified and thought part of the *phoT* gene. However, *phoU* was later found to be an independent gene by using complementation tests (2).

To better understand the basic composition of PhoU, the protein was purified. To do this, PhoU was cloned and expressed under the promoter P_L of phage lambda. Protein aggregates were found in the cytoplasm. After purifying these aggregates, the earlier established reading frame for PhoU was confirmed and the amino acid composition and the N-terminal amino acid sequence of PhoU was determined (27).

To confirm the predicted negative regulatory effect of PhoU, one group constructed a null mutation. This mutation led to constitutive, high expression of alkaline phosphatase. A plasmid containing *phoU* was added back to this mutant in an attempt to restore wild-type repression, but repression could not be restored. It was found that P_i transport in this mutant was affected. Based on this result, it was proposed that PhoU participates in P_i transport (21).

Another study contradicted some of these results (25). This study also constructed various mutants and assayed their functions. They found that the Δ *pstSCAB-phoU* and Δ *pstB-phoU* both abolished PstSCAB mediated P_i uptake. However, it was also found that the Δ *phoU* mutation alone did not affect transport. They concluded that PhoU does

not play a direct role in P_i transport through PstSCAB. It was also found that when *phoU* was the only gene deleted, there was poor growth of the cells. Seeing this problem only in the $\Delta phoU$ and that it was alleviated when *pstSCAB* or *pstB* were removed, they concluded that the poor growth is due to constitutive expression of a functional PstSCAB in the absence of PhoU. There was constitutive expression of alkaline phosphatase and repression was restored by adding back *phoU* carried on a λ phage. Throughout this study they found that in *phoU* mutants, compensatory mutations quickly accumulated in the *pstSCAB*, *phoB*, and *phoR* genes. It is thought that the conflicting results from the prior study may be due to using a mutant strain that had accumulated some of these mutations. This would explain the conflicting results that PhoU affected P_i transport (25).

Using tightly regulated gene control with arabinose and rhamnose promoter fusions helped to confirm the results in the last study. It was seen that using the tightly regulated promoters to control *phoR* or *phoB* expression, poor growth defects caused by $\Delta phoU$ were overcome. It was concluded that the poor growth was due to high-level Pst synthesis in the absence of PhoU due to the autogenous expression control of PhoB and PhoR (12).

Recently, Kim *et al.* (18) solved the crystal structure of a PhoU homologue found in *Thermotoga maritima* (Figure 2). This gave rise to some interesting results. PhoU is a symmetrical protein that has two, three-alpha helix bundles. Bound to these bundles, they found a trinuclear and a tetranuclear iron cluster. These clusters were found between conserved E(D)XXXD motif pairs. It is pointed out that the structural fold between the two sets of alpha helices is a unique, novel structural fold in the protein data bank. It is also noted that the coordination of the iron clusters by an E(D)XXXD pair has also not

been described before. Other proteins that contain similar iron clusters are all involved in iron transport or metabolism. This paper predicts that these iron clusters may act as a cofactor in some PhoU enzymatic function, such as P_i metabolism (18).

These results have not been confirmed in native PhoU from *E. coli*. It is also noted that the PhoU that was crystallized (*TM1734*) is not located in association with a *pstSCAB* operon. It is found in a gene cluster with several hypothetical genes. However, these results may be significant because the *E. coli* PhoU contains the same highly conserved amino acid regions. Future work will involve confirming the iron binding of PhoU in *E. coli* and determining the potential role the iron plays in its function (Figure 2).

There are other highly conserved regions in PhoU as well. I hypothesize that these regions are essential for proper function of the PhoU protein in repression of the Pho regulon. To test this hypothesis, several *phoU* point mutations have been constructed and tested for function (Figure 2).

Materials and Methods

Media

To grow cultures, LB growth medium (Becton Dickinson, Sparks, MD) or MOPS minimal medium (22) (FisherBiotech, Fair Lawn, NJ) were used. LB medium was sterilized by autoclaving and MOPS minimal medium was sterilized by filtration. MOPS minimal medium was used at a 1X concentration (40 mM MOPS, 4 mM Tricine, 50 mM NaCl, 9.52 mM NH₄Cl, 0.523 mM MgCl₂ 6H₂O, 0.276 mM K₂SO₄, 0.01 mM FeSO₄, 0.5 μM CaCl₂, 0.0005% Thiamine-HCl, 0.4% Glucose, 0.2 mM KH₂PO₄ [5 mM for high P_i]). Chloramphenicol (40 μg/ml), Kanamycin (50 μg/ml) (International Biotechnologies Inc., New Haven, CT), and Ampicillin (100 μg/ml) (FisherBiotech, Fair Lawn, NJ) were used. 5-Bromo 4-Chloro 3-Indolyl Phosphate, p-Toluidine Salt (X-Phos) was used by adding 1 ml of 20 mg/ml in N, N,-Dimethyl Formamide to each Liter of agar media for plates (EM Science, Guilmore, NJ).

Bacterial Strains

For reconstructing the *pstSCAB-phoU* operon, *E. coli* strain BW26337 (Δ *pstSCAB-phoU*) and BW25113 as a wild-type control were used (8). SG1, a Δ *pstSCAB-phoU*, Δ *phoB-phoR* strain was constructed by transducing the Δ *phoBR::kan* mutation from ANCH1 (36) into the BW26337 strain using P1 *clr* and standard techniques as described below (20). BW26337 was cultured in 3 ml of LB overnight. Cells were collected by centrifuging at 7500 RPM for 5 min and resuspended in 3 ml of MC buffer (0.1 M MgSO₄, 0.005 M CaCl₂). Then 0.1 ml of cells and 0.1 ml of ANCH1

P1*clr* lysate were added to a tube, 0.2 ml of cells and 0.2 ml of lysate to a second tube, and 0.2 ml cells and 0.1 ml lysate to a third tube. The tubes were placed in the 37° C water bath for 20 min. 0.2 ml of citrate buffer (9.6 g citric acid, 4.4 g of NaOH, dH₂O to 500 ml; the pH was adjusted to 5.5 using 10 N NaOH and sterilized by autoclaving) was added to each tube, then 200 µl were plated onto LB plates containing Kanamycin and placed in the 30° C incubator. A colony was isolated from the plates and the genotype was confirmed using PCR with PhoU and PhoB primers Table 1. A colony was suspended into 50 µl of water and vortexed for 1 min, then placed in a boiling heat block for 5 min, vortexed for another minute and placed on ice. To run PCR, 40 µl H₂O, 1 µl Template DNA, 2 µl Primer mix (Table 1), 1 µl dNTP's, 5 µl 10X Buffer, and 1 µl Polymerase (New England Biolabs, Ipswich, MA) were used. The PCR products were confirmed by agarose gel electrophoresis (Figure 3).

Plasmid construction

The *pstSCAB* region was amplified by PCR from *E. coli* strain MG1655 chromosomal DNA using Advantage 2 polymerase (BD Biosciences, Palo Alto, Calif.) and primers PstS forward and PstB reverse (Table 1). PstS primer contains a unique *NdeI* site whose ATG sequence is in frame with the initiating codon of *pstS*. PstB primer contains a unique *KpnI* site downstream of the stop codon of *pstB*. Amplified DNA was purified with QIAquick PCR cleanup cartridge (Qiagen, Valencia, Calif.), digested with *NdeI* and *KpnI*, and cloned into pRR48, similarly digested, to create the plasmid pRRSCAB (Figure 4, Figure 5). pRR48 is ampicillin resistant and contains the *P_{TAC}* promoter and the *lacO^{id}* operator region upstream of a multiple cloning site and tightly

regulated with IPTG. The PhoU gene (Figure 6) was similarly cloned using primers PhoU forward and PhoU reverse (Table 1). The amplified DNA was digested, purified, and ligated into pKG116 to create the plasmid pKGPhoU2 (Figure 7 and 8). pKG116 is Chloramphenicol resistant and contains the positive regulatory gene, *nahR*, from the NAH7 plasmid and the *nahG* promoter upstream of a multiple cloning site and from which expression can be controlled with sodium salicylate (38, 39). pRR48 and pKG116 are derivatives of pCJ30 and pLC112, respectively (3).

Plasmids were constructed that contained *phoR* and *phoBR* gene inserts also using pKG116 and pRR48. Similar methods were used as previously described to amplify genes with engineered *NdeI* and *KpnI* sites using primers PhoR forward, PhoR reverse, PhoB forward, and PhoB reverse (Table 1). Both vector and PCR products were digested. The plasmid vector was treated with Antarctic Phosphatase (New England Biolabs, Ipswich, MA). The DNA was purified and the *phoB* and *phoBR* inserts were ligated into the plasmids. The four plasmids where *phoBR* genes were introduced into pRR48 were named; pSG41, pSG42, pSG43, and pSG44. The plasmid with the *phoBR* genes inserted into pKG116 was named pSG45. The plasmid where the *phoR* gene was inserted into pRR48 was named pSG46.

DNA electrophoresis

For DNA electrophoresis, 0.7% agarose gels containing ethidium bromide (~0.4 $\mu\text{g/ml}$) run at 80-95 V for 45-60 min were used to separate DNA. The gels were then imaged using a Biorad Flour-S Multimager.

Alkaline Phosphatase (AP) Assays

To assay function of the mutants, alkaline phosphatase (AP) assays were performed using strains BW25113 and BW26337 as positive and negative controls, respectively. For these assays, triplicate overnight cultures in 3 ml of LB were grown. The OD₆₀₀ of the cultures were measured. 1 ml of cells was collected by centrifugation. Cells were diluted 1:2 in 1 M Tris HCl pH 8.2, two drops of chloroform and one drop of 0.1% SDS were added. Cells were vortexed for 1 minute. 100 µL of 20 mM para-nitrophenyl phosphate were added and reactions were incubated at 37° C until the solution turned yellow. Reactions were stopped with 400 µL of 1 M KH₂PO₄ and then placed on ice. Tubes were centrifuged for 1 minute and the OD₄₂₀ of the supernatant was measured. AP units are arbitrary units that equal $(OD_{420} \times 2000) / (OD_{600} \times \text{incubation time})$. The average of the three samples is reported and the standard deviation is shown with error bars.

Mutant construction

For constructing mutations in *phoU*, the pKG116PhoU2 plasmid was used as a template. A QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) was used to make point mutations to alanine (Figure 9). In order to make these mutations, primers were designed that would allow for the following mutations: D58A, D85A, R87A, E100A, R101A, D104A, D161A, E200A, R201A, D204A, and N208A (Table 1). The primers were diluted in TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA) to a concentration of 0.1 µg/µl. For the initial PCR reaction: 5 µl 10X buffer, 2 µl pKGPhoU2 plasmid (~30 ng), 1.25 µl forward primer, 1.25 µl reverse primer, 1 µl dNTP mix, and

39.5 μ l sterile ddH₂O were combined. Then, 1 μ l Pfu Ultra HF DNA Polymerase was added and the reaction was run using the Quikchng program on a PTC-300 Peltier Thermal Cycler (MJ Research, Watertown, Mass.). For the Quikchng program: the 1st step is 95° C for 30 sec. 2nd step 95° C for 30 sec, 3rd step 55° C 60 sec, 4th step 68° C 5 min, 5th step repeat steps 2-4 sixteen times, and then held at 4° C indefinitely.

After the PCR reaction, 1 μ l *DpnI* was added to each reaction tube, mixed and centrifuged for 30 sec. The reactions were incubated at 37° C for 1 hour. Competent cells were thawed on ice and 50 μ l were aliquoted into pre-chilled tubes. After incubation, 1 μ l of the PCR reaction was added to the cells, mixed, and the tubes were then incubated on ice for 30 min. The reactions were heat shocked at 42° C for 45 sec. Tubes were then immediately placed on ice for 2 min. Next, 0.5 ml of SOC media (pre-warmed in water bath) was added and the tubes were incubated for 1 hr with shaking in 37° C. For isolated colonies, reactions were plated out onto LB/Chloramphenicol media and grown overnight at 37° C. Isolated colonies were picked to streak for isolation and two separate colonies were sequenced to confirm the mutation.

For sequencing, plasmids were isolated using a QIAprep Miniprep Kit (Qiagen, Valencia, CA). Then, 9 μ l of plasmid DNA was added to 1 μ l of 20 μ M primer (one PhoU forward sequencing primer reaction and one PhoU reverse sequencing primer reaction) (Table 1). Then the reactions were submitted for sequencing at the BYU DNA Sequencing Center. The nucleotide sequences were compared with the wild-type PhoU to screen for the proper mutation.

After confirming the proper mutation through sequencing, a culture was grown up in LB and 1 ml of culture was added to 250 μ l of 80% glycerol and stored in the -76° C freezer.

Transformations

To routinely prepare cells for transformation, a 3 ml culture of cells was grown overnight in LB growth media with the proper antibiotics added. Then this culture was diluted 1:100 into 25 ml of fresh LB growth media. This new culture was grown at 37° C with shaking until it reached an OD_{600} of $\sim 0.3-0.4$. At this point, cells were collected by centrifuging for 10 min at 5000 rpm at 4° C using the SS-34 rotor in a Sorvall RC 5C plus centrifuge. The supernatant was removed and the pellet was resuspended in 5 ml of cold 0.1 M $CaCl_2$. Then samples were incubated on ice for 15-30 min. Cells were pelleted again, the supernatant removed, and the pellet was resuspended in 0.8 ml of $CaCl_2$ (with 15% glycerol added if storing cells at -80° C). Cells were then incubated on ice for 30 min to 2 hours prior to transformation.

For the transformation procedure, 0.1 ml of competent cells was placed into a clean micro-centrifuge tube and 1-5 μ l of DNA were added to each tube (one tube was maintained without DNA as the negative control). Tubes were incubated on ice for 30 min, heat shocked in a 42° C water bath for 75 sec, and returned to the ice for 2 min. 0.9 ml of LB media were added to each tube and the tubes were incubated at 37° C for 1 hour. Various amounts of cells were spread onto agar plates containing the proper antibiotic to select for positive transformants.

Protein electrophoresis

For performing immunoblots of different samples, a 12% SDS-polyacrylamide gel was prepared. For the separating gel, 1.875 ml acrylamide (40%), 1.125 ml water, 2.97 ml of 758 mM Tris pH 8.8 0.202% SDS solution, 30 μ l 10% APS, and 3 μ l TEMED (BioRad, Hercules, CA) were added. For the stacking gel 0.581 ml acrylamide (40%), 0.194 ml water, 5.16 ml of 145 mM Tris pH 6.8 0.116% SDS solution, 30 μ l 10% APS, and 6 μ l TEMED were used. Samples were suspended in 50 μ l of Laemmli Sample Buffer (BioRad, Hercules, CA) and boiled on a heat block for 5 minutes prior to loading.

For immunoblotting, a WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit (Invitrogen, Carlsbad, CA) was used. The provided protocol for using nitrocellulose membranes was followed. For probing the membrane, 1 μ l of anti-PhoU polyclonal rabbit antiserum diluted into 10 ml of blocking solution was used.

Results

Cloning of PstSCAB and PhoU/ Reconstruction of operon

As mentioned earlier, it has been seen that a *phoU* deletion mutant leads to poor growth and accumulation of compensatory mutations in *phoBR* or *pstSCAB* genes (25). In order to genetically study PhoU, the first goal was to reintroduce the *pstSCAB-phoU* operon in BW26337 (Δ *pstSCAB-phoU*) to test different *phoU* mutations for function. The experimental design was to clone the *pstSCAB* genes separately from *phoU* on compatible plasmids. The *pstSCAB* genes were amplified by PCR and ligated into pRR48 to make pRRSCAB. Using similar methods, *phoU* was amplified and ligated into pKG116 to make pKGPhoU2 (Figure 4, 5, 7, and Figure 8).

It was found that by transforming the pKGPhoU2 plasmid followed by the pRRSCAB plasmid into BW26337, wild-type repression of alkaline phosphatase (AP) was restored when cultures were grown in LB growth medium (Figure 10). This is seen by comparing the wild-type control, the BW25113 strain, with the reconstructed system. It was also found that it was important to use freshly transformed strains to test for function.

Figure 10 is a graph of the results from AP assays of the reconstructed signaling system of cells grown in P_i-replete LB growth media. It is seen that the positive control, BW25113, is able to repress expression of AP when grown in these conditions. Next, it is noted that the negative control, BW26337, is unable to repress to wild-type levels the AP expression. However, the level of induction is not the same as a fully induced system whose AP units approach the thousands. Next, by introducing the *pstSCAB* genes on the pRR48 plasmid, repression of the system is not observed. It is also seen that when

pKGPhoU2 is introduced into the BW26337 strain, in the absence of pRRSCAB, there is also no repression. However, when both the *phoU* gene and the *pstSCAB* genes are introduced on compatible plasmids, it is seen that repression of AP expression similar to that of wild-type is restored. This figure demonstrates the successful complementation of the Δ *pstSCAB-phoU* operon of BW26337 by two compatible plasmids. It also shows that the repression of AP expression is an indicator of PhoU function in the presence of a plasmid that expresses the PstSCAB proteins.

Site Directed Mutagenesis and Rationale of Mutants

Overview of techniques

For construction of PhoU mutants, the pKGPhoU2 plasmid was used as a template. Primers were designed to amplify the plasmid with a few nucleotide changes in order to alter the selected codon to an alanine (Table 1) (Figure 2). After PCR amplification, the template plasmid was digested using *DpnI* and the mutant plasmids were transformed into *E. coli* cells. The plasmids were prepared and the *phoU* region was amplified and sequenced in order to confirm the proper nucleotide changes and to screen for any other changes from the *phoU* wild-type sequence. The plasmids that showed the proper mutations were then tested for function.

Rationalization of mutants choice

Amino acid residues within PhoU were selected for mutation based upon conserved sequences found in various BLAST search alignments of PhoU homologues

(Figure 11). Highly conserved amino acids were selected for mutation (Figure 2). Many of these amino acids are charged in nature (aspartic acid, arginine, and glutamic acid). Changing these amino acids to a small, uncharged amino acid would change the nature of the residue and possibly disrupt side chain mediated interactions having a low probability of disrupting structure. For this reason, it was chosen to mutate these residues to an alanine. The methyl side chain of alanine would not be predicted to sterically hinder the formation of secondary and tertiary structures.

Solving the structure of PhoU in *T. maritima* made the choice of these regions even more interesting (18). It was found that many of our highly conserved residues fell within the predicted iron binding sites of PhoU (Figure 2). However, others fall outside these regions. Based upon the level of conservation of all these residues, it is hypothesized that they are essential for function. It is possible that different residues play different roles in allowing PhoU to function.

It was found that another plasmid, pKGPhoU1, contained a semi-conserved mutation, V128A, in the predicted PhoU sequence. To eliminate the possibility that this mutation would be the reason for phenotypic changes, pKGPhoU2 was used to construct the mutants. Two double mutants were also constructed and confirmed through sequencing, D161A/D104A and N208A/D104A.

Analysis of Mutants

Western blot

In order to confirm the production of stable protein from the *phoU* mutants, samples were collected from cultures of BW26337 pRRSCAB with the mutant plasmids

to test for the presence of PhoU using immunoblot analysis. For the immunoblot, cells were resuspended in SDS-PAGE loading buffer. The samples were then electrophoresed using SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and were probed using rabbit polyclonal anti-PhoU antibodies. The membrane was then treated with a secondary antibody and a chemiluminescent substrate. The membrane was then exposed to x-ray film which was then developed (Figure 12).

There is a strong signal for PhoU in the positive control of BW26337 pKGPhoU2 pRRSCAB. It is also seen that the negative control, BW26337 pKG116 pRRSCAB, shows no PhoU product. Many of the mutants produced stable PhoU proteins of similar size. However, there was little or no stable protein produced from the D104A, D161A, D161A/D104A, and N208A/D104A PhoU mutants. What causes this instability is unknown. It may be that the proteins are targeted for rapid degradation. It may also be possible that for some reason no PhoU protein was produced. Given the lack of stable protein in these samples, these mutants are excluded from further testing.

Test of Dominance

To test for potential dominance of these mutants, they were transformed into the wild-type strain, BW25113, and assayed for AP expression in LB growth media. A dominant mutation would be predicted to result in unrepressed levels of AP. A dominant mutant would allow for further tests of the function of PhoU. Determining the mechanism of a dominant mutant would shed light on the mechanism by which PhoU functions.

Figure 13 contains a chart of results of this assay. It is seen that when grown in LB growth media without any added inducer (salicylate) that the wild-type strain, BW25113 shows repression of the system. It is also seen that in the BW26337 strain that repression is lost. In these conditions, there is no loss of repression in any of the PhoU mutants showing that none of these mutants have a dominant negative phenotype.

In order to test if higher expression of the plasmid would affect the ability of the strain to repress expression in LB growth media, these strains were tested at two different levels of added inducer (750 nM and 1500 nM salicylate) (Figure 14 and Figure 15).

No significant loss of function with salicylate added up to a 1500 nM concentration is observed (Figure 15). The only mutant that appears to lose some repression was D85A. However, the p-value of a t-test between BW25113 and the D85 strain on the 1500nM salicylate assay is 0.2235, which fails to meet an alpha value of 0.05. With this it is concluded that none of the selected mutants have a dominant negative phenotype in these conditions.

Complementation tests for mutant function

To test mutants for the ability to restore wild-type repression of AP expression when grown in LB growth media, AP assays were performed on the reconstructed systems with the mutant plasmids placed in the BW26337 pRRSCAB background (Figure 16). These assays used triplicate cultures of each sample from freshly transformed cells. These assays were repeated to confirm the results and the results are combined to calculate the average and standard deviation of each sample. All error bars represent one standard deviation.

The graph in Figure 16 shows the results from the tests on the PhoU mutants. It is seen that the controls functioned properly. The positive control is the BW25113 strain which, as the wild-type, shows repression of AP expression. The negative controls are the BW26337 strain which lacks repression as well as BW26337pKG116pRRSCAB, which also lacks repression and shows that any repression seen is due to the PhoU protein and is not based on the pRRSCAB plasmid. With the mutants, it is seen that some are able to repress AP expression while others do not. From these results, it can be concluded that the D58A, D85A, R87A, E100A, R101A, R201, and N208 strains lose some or all repressive function. It is also seen that the E200A and D204A mutants have either little or no effect on PhoU's ability to repress the system.

Discussion

PhoU plays an important role in signaling to PhoR in high P_i environments. This leads to repression of Pho regulon genes. The two main hypotheses on how this signaling is mediated are: 1. PhoU interacts in a protein complex to transfer the signal, perhaps through conformational changes between proteins, and/or 2. PhoU acts as an enzyme and is able to transfer the signal through its enzymatic role, perhaps by creating a second messenger that is sensed by PhoR. The work reported in this thesis neither confirms nor eliminates either possibility, but provides important foundational information for the further study of PhoU. The mutants constructed in this study may be tested in the future to help better define the mechanism of signal transduction.

In constructing the complementation system, it was seen that freshly transformed strains were better able to restore repression. This may be due to different mutations accumulating over time. These accumulated mutations were seen when others attempted to generate *phoU* deletion mutants. Seeing loss of function over time may indicate that there is still some selective pressure on cells in our reconstructed system. This may indicate that there is some other function in the PstSCAB and PhoU proteins that is not complimented with our reconstructed system. It may also be due to some intrinsic time sensitivity found in our system. Due to the unknown nature of PhoU, it is difficult to rule out the possibility of other, independent, functions of PhoU.

We see that some of these highly conserved regions of PhoU are essential for proper signaling to repress AP production in LB growth media. It is interesting to note that there were no dominant negative mutants found. A dominant negative mutant may indicate protein-protein interaction. However, the lack of a dominant negative mutant

does not eliminate the possibility of protein-protein interactions. It is also interesting to note that a strain with a *pstS* mutant that lacks P_i transport through the Pst transporter loses proper expression control. However, this control can be restored when PitA or PitB is overexpressed (16). It is possible that the Pst system can sense P_i concentration independent of its transport function, or this may implicate that PhoU somehow senses P_i concentration independent of a protein complex or that it can complex with the Pit proteins as well as the Pst system.

The presence of iron in the crystal structure of PhoU from the *T. maritime* raises important questions. Iron is an important co-factor in many enzymes. Some of the mutants in the putative iron binding domains lead to loss of function (D58A, E100A, R101A, and R201A). However, other mutants in the putative iron binding domains did not disrupt the repression of the system (E200A and D204A). While the E200A mutation does not lead to loss of function, E100A does. These are symmetrical residues from each of the iron binding regions. One would assume that they have similar function, but this is not seen. Perhaps one of the iron binding regions is more important for proper signaling than the other. It is important to note that the PhoU protein in *E. coli* has not been shown to bind iron. If iron binding in the *E. coli*'s PhoU is found, it would be interesting to see which PhoU mutants bind iron and which do not. If loss of function correlates with iron binding, it could be concluded that the iron is essential for proper signaling. The next step would then be to look for the function of iron in this protein. Is it essential for proper protein confirmation? Does it mediate protein-protein interaction or is it an enzymatic cofactor? This would shed light on the function of iron in PhoU and signaling P_i sufficiency.

In comparing the sequences of PhoU in *E. coli* with that of *T. maritima*, it is seen that in *E. coli*, each of the putative iron binding boxes contain an Arg/Lys residue after the first conserved Glu/Asp. This is not seen in the *T. maritima* sequence, where the first Glu/Asp of each box is followed by Glu, Asn, Ser, and Ile respectively. Given the basic nature of the conserved Arg/Lys residues, it is unlikely that they help to mediate iron binding. Perhaps these residues allow PhoU in *E. coli* to bind to another metal, metal phosphate, or metal nucleotide groups. The importance of these residues in PhoU is demonstrated by the loss of function with both the R101A and R201A mutants. This is even more interesting to note, given that the D200A and D204A mutants maintained function. That would imply that the basic side group of R201 is more important in PhoU function than either of the acidic side groups found in D200 and D204. The possibility of PhoU binding another metal, metal phosphate, etc. raise many more questions and give rise to theories on the ability of PhoU to sense and pass on P_i signals to PhoR.

Future work looking at the localization of PhoU may also shed light on its function. PhoU localizes to the membrane in wild-type cells. It would be interesting to see if there is a correlation between the function of these mutants and their localization. If interaction with membrane bound proteins were essential, then we would expect that any non-membrane localizing mutants would lose function. However, if PhoU can act as an enzyme it may be independent of cellular localization, then functional mutants need not localize to the membrane.

The mutants outside of the predicted iron binding regions also lead to loss of function (D85A, R87A, and N208A). The N208A mutation is near one of the putative iron binding sites and may assist in that function. The D85A and R87A mutations are at

the end of an alpha helix between the first two putative iron binding regions. These regions may be essential to allow for proper protein folding or conformation of PhoU. An alanine mutation would not be predicted to disrupt an alpha helix like a proline mutation would. However, it may disrupt essential side chain interactions that allow for proper confirmation. It is also possible that this region is essential for protein-protein interaction. These mutations could then lead to loss of interaction and failure to signal.

It would be interesting to see if any of these mutants were able to restore repression in a system of overexpression of PitA or PitB. As mentioned before, when PstS was mutated, overexpression of PitA or PitB was able to restore proper signaling of the Pho regulon (16). It is possible that the mutation that inhibits repression in this system is independent of the signal that is mediated by PitA and PitB overexpression. Similar results in the overexpression of PitA or PitB would implicate that the method of signal transduction in this system is singular. However, if there were differences between the results, it may imply that there are multiple signal transduction pathways that act to control this system. This hypothesis may correlate with the idea of cross-regulation of the Pho regulon. Perhaps, PhoU has a signaling function to the Pho regulon as well as an independent enzymatic function in P_i metabolism (25). We see that the transport and signaling functions found in the PstSCAB transporter can be independent of one another. More study of this system is needed to answer these and many other questions.

This work has been important to develop a complementation system to test for PhoU function. It has also been important to show that indeed some of the highly conserved regions of PhoU, when mutated to alanine, lose proper signaling function. It shows that other mutations to alanine do not disrupt the signaling function of PhoU.

Future study of PhoU and these mutants may shed light on the method of signaling used by PhoU. Finding distinct differences between the functioning and nonfunctioning mutants can help to distinguish the method of signaling. Learning about signaling in this system may be applied to other two-component systems. This may also give rise to new antimicrobials that target these systems.

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Tables

Primer name	Sequence in 5'-3' direction
PhoB forward	GCATCACATAT <u>G</u> GCGAGACGTATTCTGGTCGTAGAAGATG
PhoB reverse	GTC <u>GGTACCT</u> TAAAAGCGGGTTGAAAAACGATATCCTG
PhoR forward	GCATCACATAT <u>G</u> CTGGAACGGCTGTCGTGGAAAAGGCTGG
PhoR reverse	GTC <u>GGTACCT</u> TAATCGCTGTTTTTGGCAATTAAACGTTC
PstS forward	GCATCACATAT <u>G</u> AAAGTTATGCGTACCACCGTCGCAACTG
PstB reverse	GTC <u>GGTACCT</u> CAACCGTAACGACCGGTGATGTAGTCTTC
PhoU forward	GCATCACATAT <u>G</u> GACAGTCTCAATCTTAATAAACATATTTTC
PhoU reverse	GTC <u>GGTACCT</u> TATTTGTCGCTATCTTTCCCCGCCAGCAG
PhoU forward	ACATATTTCCGGCCAGTTCA
PhoU reverse	GCCAGCAGTTTATCCAGCTC
D58A forward	CGCGTCATCGAAGGCG C AAAGAACGTCAACATGATGG
D58A reverse	CCATCATGTTGACGTTCTTT G CGCCTTCGATGACGCG
D85A forward	CCGACGGCGAGCG C ACTGCGACTGGTTA
D85A reverse	TAACCAGTCGCAGT G CGCTCGCCGTCGG
R87A forward	CGGCGAGCGACCTGG C ACTGGTTATGGTGA
R87A reverse	TCACCATAACCAGT G CCAGGTCGCTCGCCG
E100A forward	CCATTGCCGAGCTGG C TCGTATTGGCGACG
E100A reverse	CGTCGCCAATACG A GCCAGCTCGGCAATGG
R101A forward	ATTGCCGAGCTGGAG G CAATTGGCGACGTGGCGG
R101A reverse	CCGCCACGTCGCCAATT G CCTCCAGCTCGGCAAT
D104A forward	TGGAGCGTATTGGCG C AGTGGCGGACAAAATCTG
D104A reverse	CAGATTTTGTCCGCC A T G CGCCAATACGCTCCA
D161A forward	GCGGTACGTATTTATCGTGAAG C AAAAAAGTCGATCAGG
D161A reverse	CGTATTCCTGATCGACTTTTTTT G GCTTCACGATAAATACGT
E200A forward	GCGCGTTCTATCG C ACGTATTGGCGACC
E200A reverse	GGTCGCCAATACGT G CGATAGAACGCGC
R201A forward	GCGCGTTCTATCGAAG C AATTGGCGACCGCTGCC
R201A reverse	GGCAGCGGTCGCCAATT G CCTTCGATAGAACGCGC
D204A forward	CTATCGAACGTATTGGCG C ACGCTGCCAGAATATTTGTGA
D204A reverse	TCACAAATATTCTGGCAGCGT G CGCCAATACGTTTCGATAG
N208A forward	TATTGGCGACCGCTGCCAG G CAATTTGTGAGTTTATCTTCT
N208A reverse	GTAGTAGAAGATAAACTCACAAATT G CCTGGCAGCGGTCCG
N208A reverse	GTAGTAGAAGATAAACTCACAAATT G CCTGGCAGCGGTCCG

Table 1. List of PCR primers used in this study.

Start codons are underlined. The engineered **NdeI/KpnI** sites are labeled accordingly.

Nuclotide changes in the mutagenesis primers are labeled in bold.

Figures

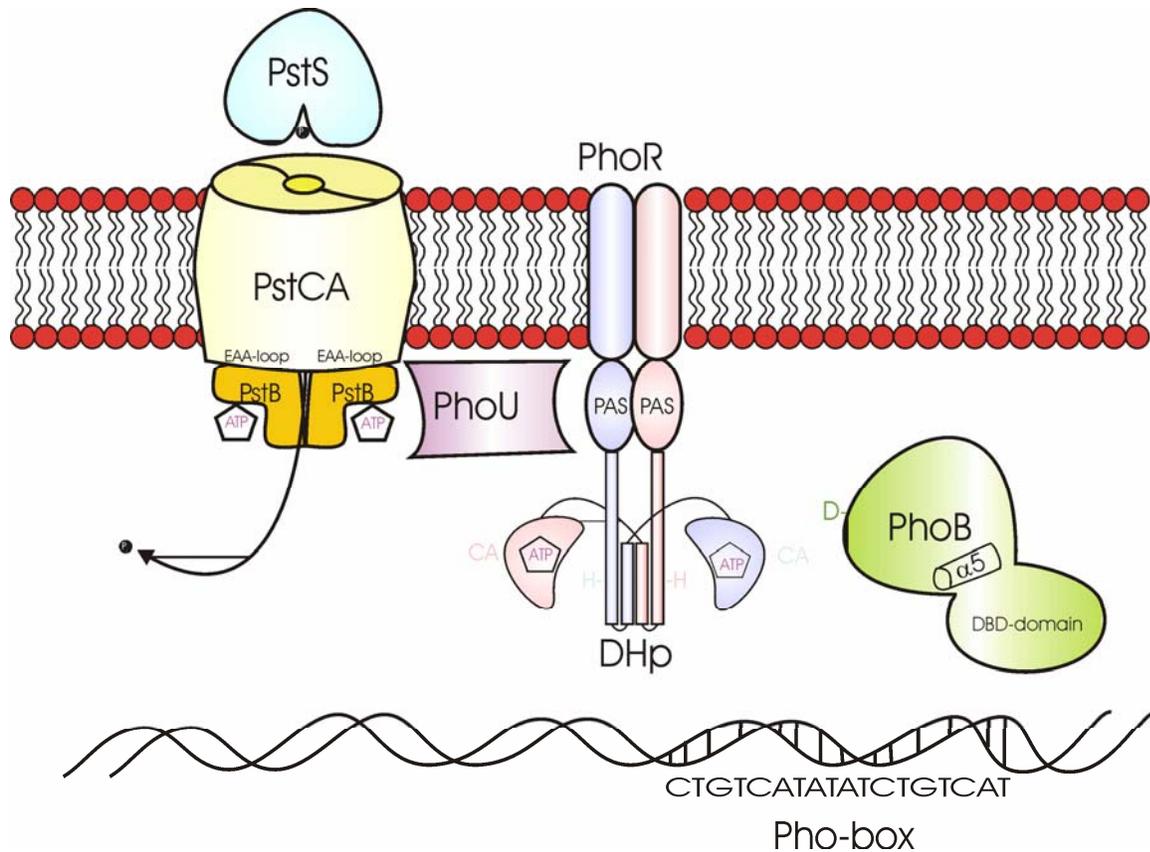


Figure 1. Overview of Pho regulon signaling components.

This is an overview of the major components in signaling for gene regulation of the Pho regulon. PhoR is the histidine kinase that autophosphorylates upon receiving a signal. PhoB, the response regulator, is activated by phosphorylation. It then activates expression of the Pho regulon by binding to Pho-boxes. PstSCAB is a phosphate specific ABC transporter complex. PhoU is a negative regulator of the Pho regulon in phosphate-replete conditions. PstSCAB and PhoU are important in regulating expression control of the Pho regulon.

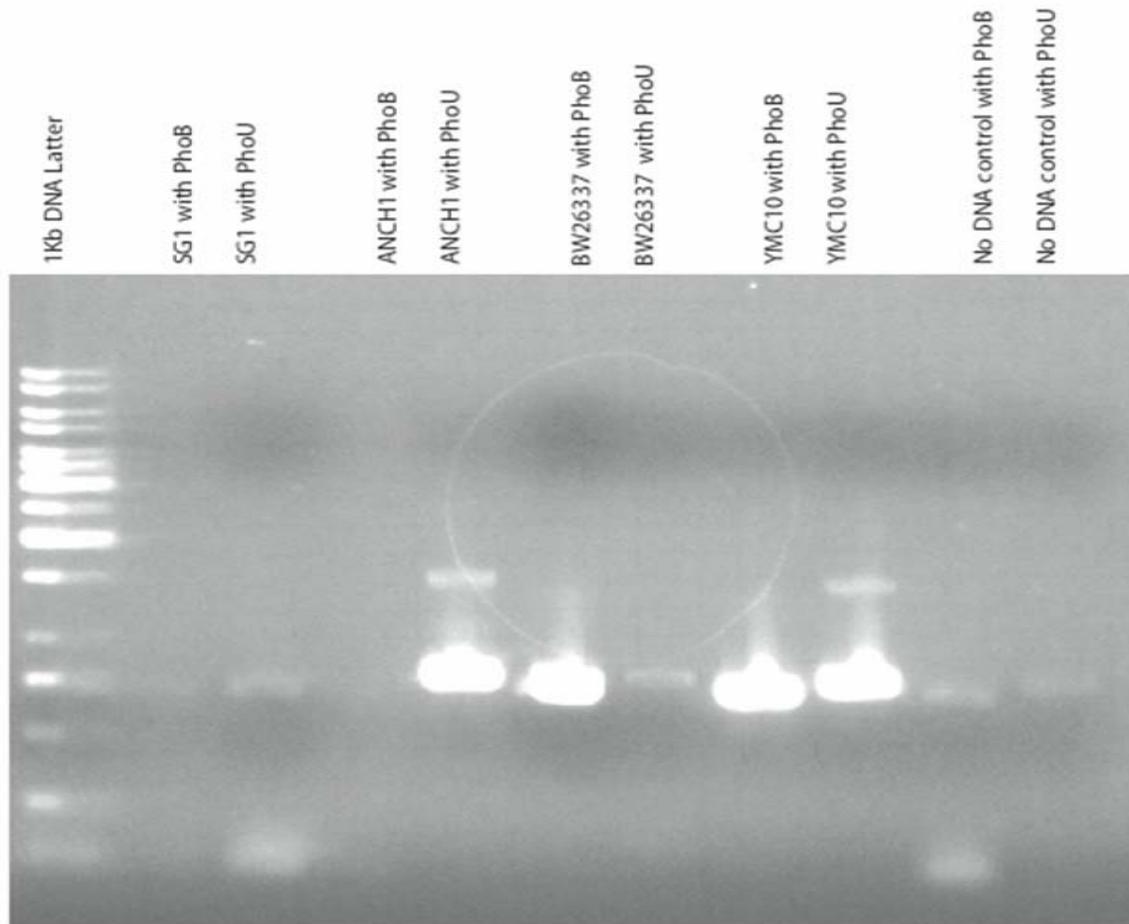


Figure 3. SG1 confirmation gel.

This shows results of an agarose gel electrophoresis performed on the PCR products of various reactions using primers for PhoB and PhoU (Table 1). In this figure, the controls are the wild-type strain YMC10, the $\Delta pstSCAB-phoU$ strain BW26337, and the $\Delta phoBR$ strain ANCH1. The PCR reactions were specific to the genotype of the controls. It is seen that SG1 contains the proper genotype ($\Delta pstSCAB-phoU \Delta phoBR$).

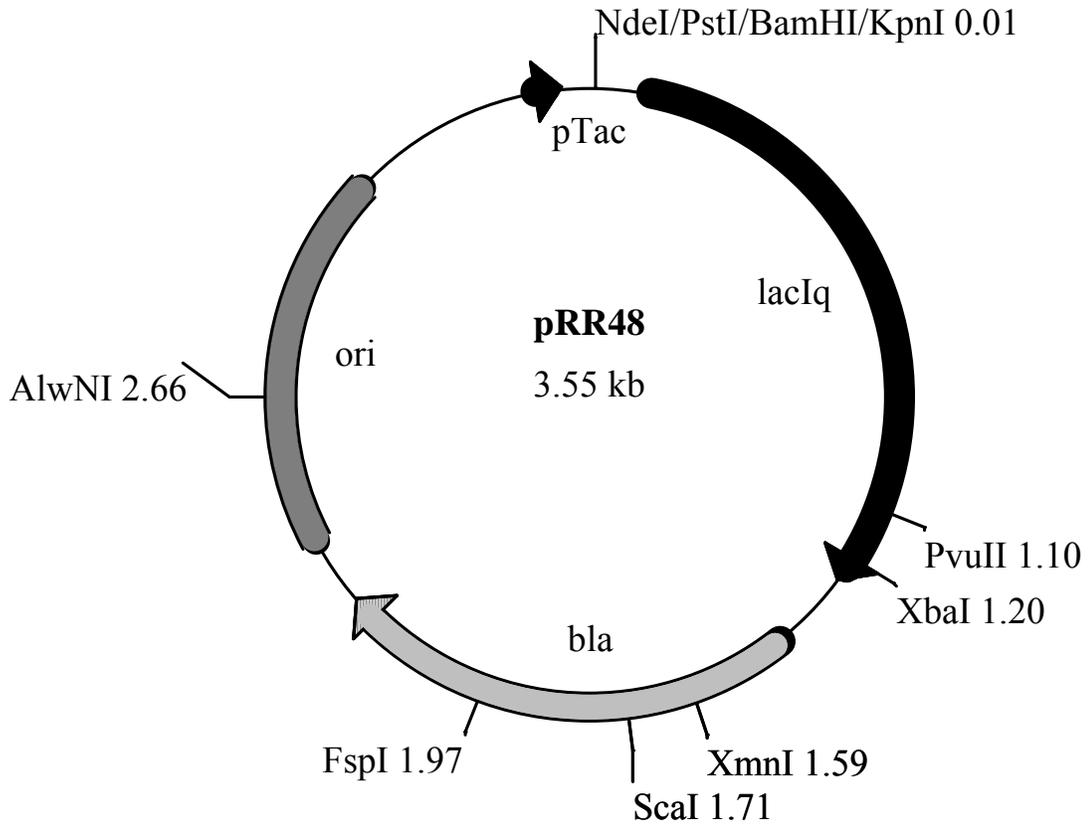


Figure 4. Plasmid map of pRR48.

This is a plasmid map of pRR48, plasmid derivative of pCJ30 (3). pRR48 is ampicillin resistant and contains the P_{TAC} promoter and the $lacO^{id}$ operator region upstream of a multiple cloning site so that expression is tightly regulated with IPTG.

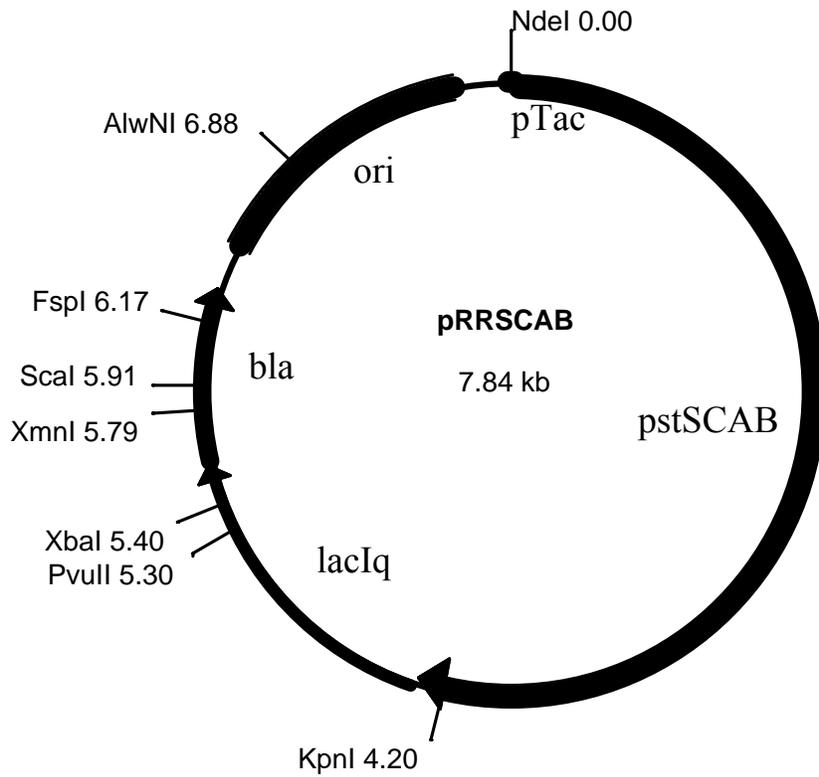


Figure 5. Plasmid map of pRRSCAB.

This plasmid was constructed by inserting the *pstSCAB* genes into pRR48 plasmid between the *NdeI* and *KpnI* sites of the multiple cloning site.

ATGGACAGTCTCAATCTTAATAAACATATTTCCGGCCAGTTCAACGCCGAACT
GGAAAGTATCCGCACGCAGGTGATGACCATGGGCGGCATGGTGGAGCAGCA
GCTTTCTGATGCAATCACCGCGATGCATAACCAGGACAGCGATCTGGCGAAG
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GAAGCCTGCGTGCGCATTATCGCCAAACGTCAGCCGACGGCGAGCGACCTGC
GACTGGTTATGGTGATCAGTAAAACCATTGCCGAGCTGGAGCGTATTGGCGA
CGTGGCGGACAAAATCTGCCGTACTGCGCTGGAGAAATTCTCCCAGCAGCAT
CAGCCGTTGCTGGTAAGTCTGGAGTCGCTGGGCCGTCATACCATCCAGATGCT
GCACGACGTGCTGGACGCGTTCGCGCGGATGGACATTGACGAAGCGGTACGT
ATTTATCGTGAAGATAAAAAAGTCGATCAGGAATACGAAGGTATTGTTCGTC
AACTGATGACCTACATGATGGAAGATTCGCGTACCATTCCGAGCGTACTTACT
GCGCTGTTCTGCGCGCGTTCTATCGAACGTATTGGCGACCGCTGCCAGAATAT
TTGTGAGTTTATCTTCTACTACGTGAAGGGGCAGGATTTCCGTCACGTCGGTG
GCGATGAGCTGGATAAACTGCTGGCGGGGAAAGATAGCGACAAATAA

Figure 6. Gene sequence of *phoU*.

PhoU nucleotide sequence in *Escherichia coli* with the highlighted Forward sequencing primer and Reverse sequencing primer sites.

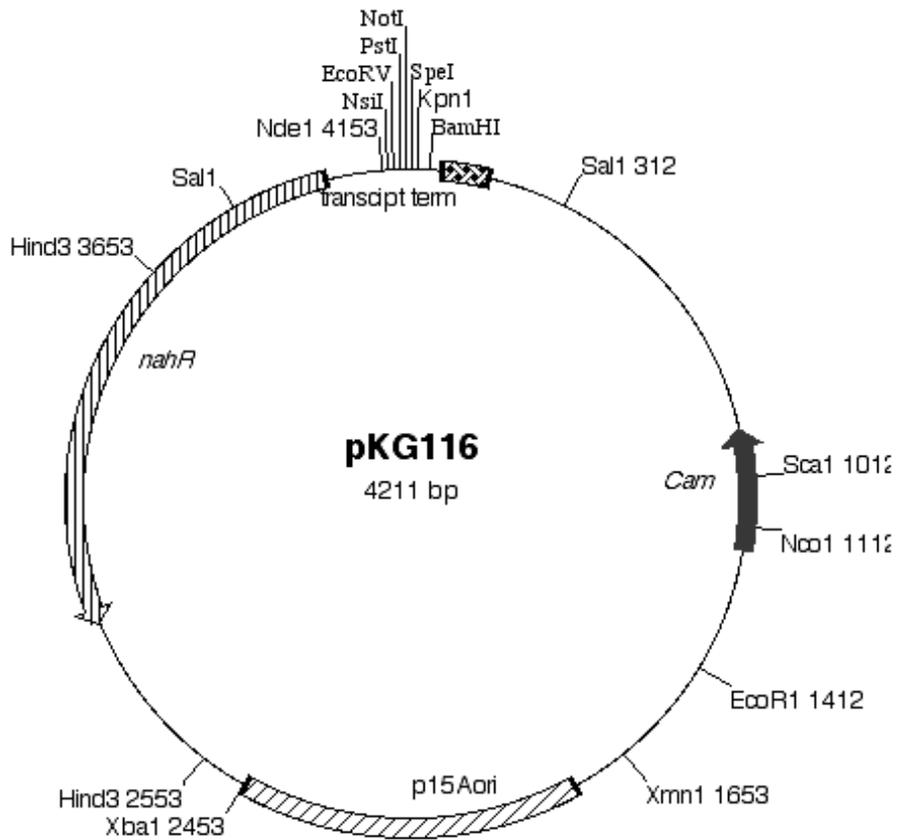


Figure 7. Plasmid map of pKG116.

This figure shows the plasmid map of pKG116. pKG116 is a plasmid derivative of pLC112 (3) that is chloramphenicol resistant and contains the positive regulatory gene, *nahR*, from the NAH7 plasmid and the *nahG* promoter upstream of a multiple cloning site and from which expression can be controlled with sodium salicylate (38, 39).

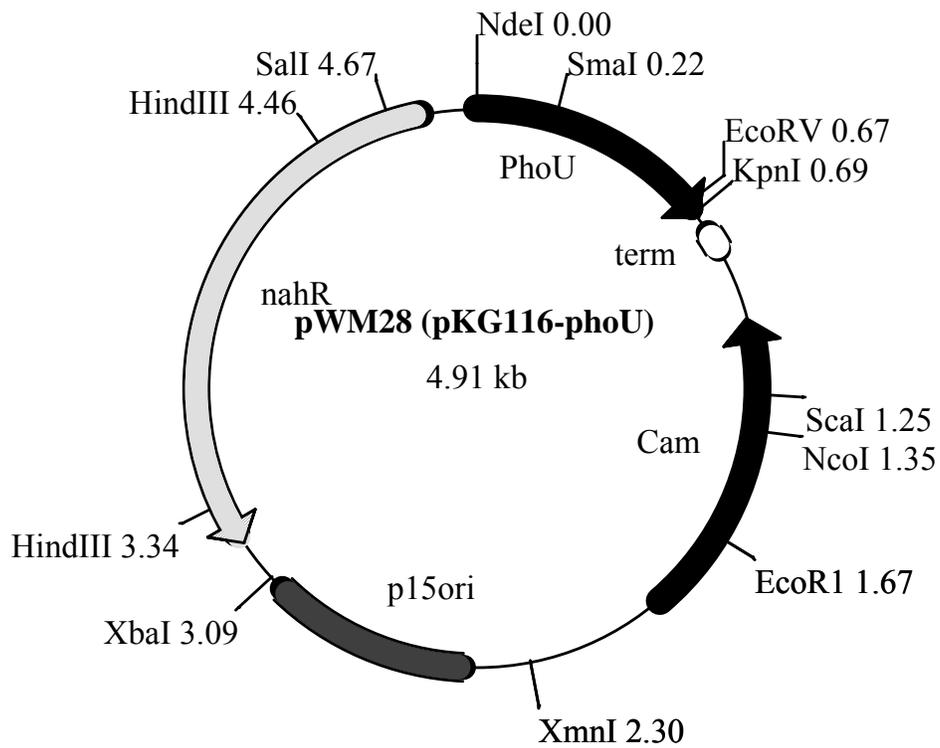


Figure 8. Plasmid map of pKGPhoU2.

This is a plasmid map of the plasmid referred to in this thesis as pKGPhoU2. This is a plasmid that was constructed by inserting the *phoU* gene into the pKG116 multiple cloning site between the *NdeI* and *KpnI* sites.

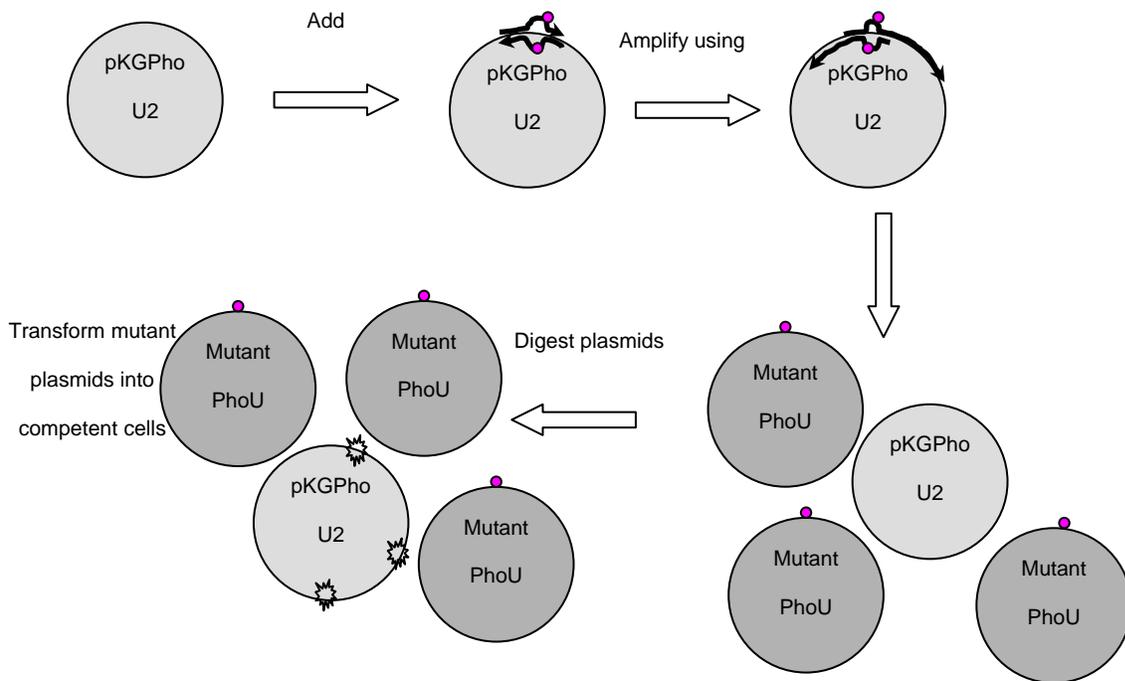


Figure 9. Mutagenesis overview.

This was the process of creating plasmids with site directed mutations in the *phoU* gene. Using the pKGPhoU2 plasmid as a template, PCR reactions were performed using primers designed with nucleotide changes in the codon for a highly conserved amino acid that lead to an alanine codon. After PCR, the sample is treated with *DpnI*, an endonuclease that only cleaves methylated DNA. This treatment leads to the cleavage of any original, unmutated plasmid DNA.

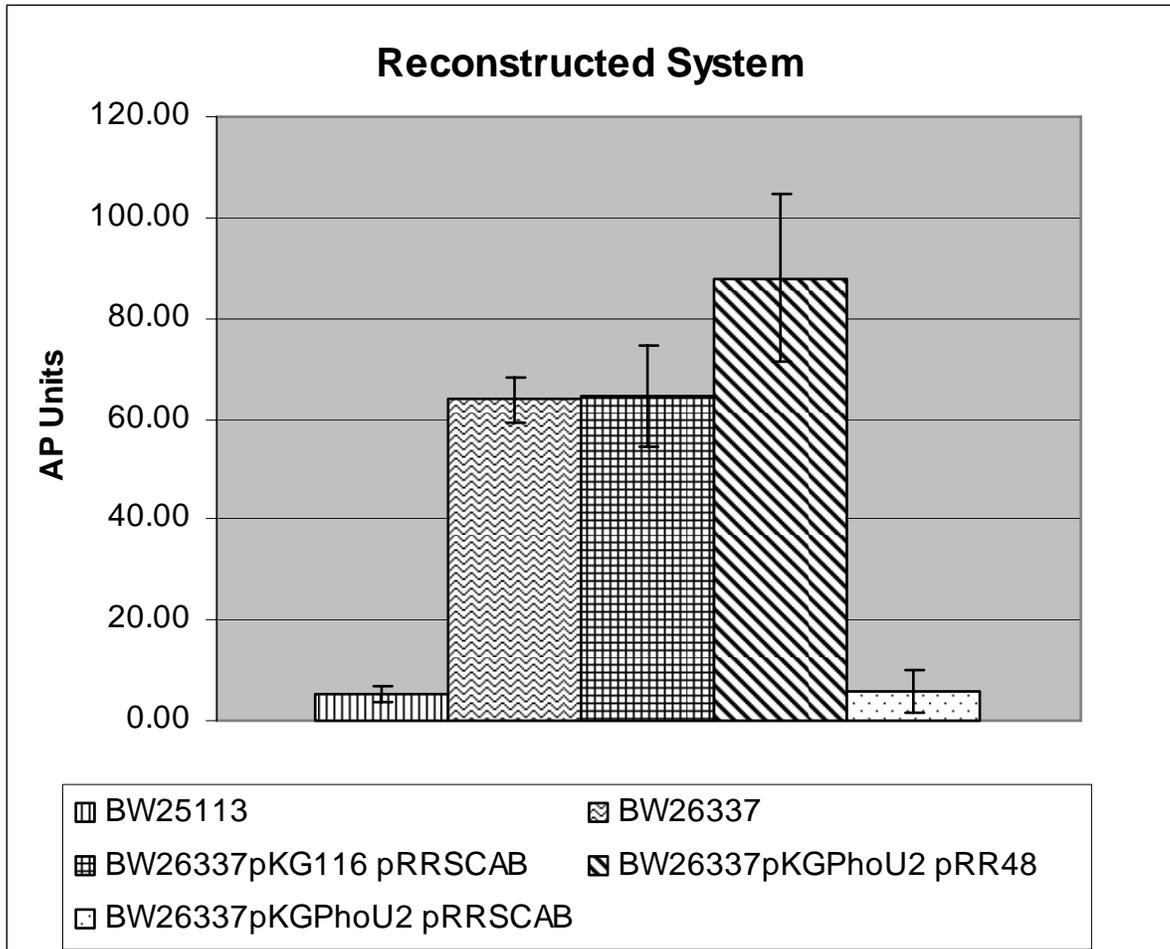


Figure 10. Confirmation of repression in reconstructed system.

The AP assay results confirming complementation of signaling when cells were grown in LB growth media. It was seen that with the wild-type strain, BW25113, there is repression of the Pho regulon. This repression is also seen in the reconstructed system of BW263337 pKGPhoU2 pRRSCAB. The negative control of the BW26337 strain shows lack of repression. The controls of BW26337 pKG116 pRRSCAB and BW26337 pKGPhoU2 pRR48 show that repression is only regained with pRRSCAB and pKGPhoU2 combined together and that this repression is not an independent activity of either plasmid.

```

PhoU E. coli
sp |032488| PHOU_ENTCL
tr |07CPE7
tr |08CZF8
tr |06CYN4
tr |083WB2
tr |07N9U3
tr |07MNI6
tr |082TX5
tr |08XZ71
sp |09XBG0| PHOU_BURSP
tr |06FFB4
sp |052989| PHOU_RHIME
sp |067053| PHOU_AQUAE
tr |0971D9
tr |072GJ7
sp |09PBJ9| PHOU_XYLFA
tr |0927Z9
tr |072D45
sp |09RS84| PHOU_DEIRA
tr |088YK6
tr |08CPA2
tr |08E5K7

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----MDSLNLNKHISGQFNABELESIRTVQVMTMGGMVEQQLSDAITAMHNQDSELAQRVIEGDKVNMMEVAID EACVRI IAKRQP--TASDLRLVMAI IKTIAELERIGD VADKICRTALE
----MDNLSLNLNKHISGQFNABELESIRTVQVMTMGGMVEQQLSDAITAMHNQDSELAQRVIEGDKVNMMEVAID EACVRI IAKRQP--TASDLRLVMAI IKTISELERIGD VADKICRTALE
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-----MSDKHLSTQFDLTLSTIKVQLMGGMVEQQLSDAITAMHNQDSELAQRVIEGDKVNMMEVAID EACVRI IAKRQP--TASDLRLVMAI IKTISELERIGD VADKICRTALE
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-----MNTSYNHYI VKS YDDLELNRLVTEIIRMGIAVAQLEAALDVVERRDDKAADR IVMNDEAIDALEQSI SHDMVRL-ALRGP--MARDLRLVMAI IKTISELERIGD VADKICRTALE
-----MVVRKIFTEQLNDLHQHLMEMGLMELANEAI FKAVKNDLSVHRDTELAKQVVAEDKAI INNMELSLERQSFELIALQ--VGMDLRLVMAI IKTISELERIGD VADKICRTALE
-----METHFHRELQKLRITLLEMAHAHTTAIEDATTALIRRDVDSLASKVAGDRHINAL ECDVD--ERSLRLVMAI IKTISELERIGD VADKICRTALE
-----MREALEADLRAVLNGLANMLGTVERMLPVAEAVLLHERADLLEGEVRS LDREVAQEAQLEAECLRI IALHQ--VARDLRLVMAI IKTISELERIGD VADKICRTALE
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unk |VIRT8996| Blast_submission
sp |032488| PHOU_ENTCL
tr |07CPE7
tr |08CZF8
tr |06CYN4
tr |083WB2
tr |07N9U3
tr |07MNI6
tr |082TX5
tr |08XZ71
sp |09XBG0| PHOU_BURSP
tr |06FFB4
sp |052989| PHOU_RHIME
sp |067053| PHOU_AQUAE
tr |0971D9
tr |072GJ7
sp |09PBJ9| PHOU_XYLFA
tr |0927Z9
tr |072D45
sp |09RS84| PHOU_DEIRA
tr |088YK6
tr |08CPA2
tr |08E5K7

KFSQQ--HQPLL--VSLES LGRHTVQMLHDVLD AFARMDLDEAVRIYREDKVV DQ EYEGIVRQLM TYMME DSRTIPSVLTALFCARSI ERIGDRCONICEYIFYYVKGQDFRHVGGDEL DKL LAGK-DS----
KFSQQ--HQPLL--VSLES LGRHTVQMLHDVLD AFARMDLDEAVRIYREDKVV DQ EYEGIVRQLM TYMME DSRTIPSVLTALFCARSI ERIGDRCONICEYIFYYVKGQDFRHVGGDEL DKL LAGK-DPKE---
KFSQQ--HQPLL--VSLES LGRHTVQMLHDVLD AFARMDLDEAVRIYREDKVV DQ EYEGIVRQLM TYMME DSRTIPSVLTALFCARSI ERIGDRCONICEYIFYYVKGQDFRHVGGDEL DKL LAGK-DPKE---
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SPSSK--ERQFN--VSLEPLARQAIQMLHLVLD AFARMDVDAAEVHKMDDKI DAEYEAIVRQLM TYMMEKNIPNI I QVMWSARA IERVDRCONICEYIIFYYVKGQDFRHVGGDEL DKL LAGK-INDALR-----
IYAA-DRMYTPR-FVEIRHIAAIAMDLR KALDAFARVDPNASAE IVRQDEQV D EEFRSIIRHLVTFMME DPKRISTATEILFAAKAV ERIGDHAKNMSEYVVMYVKGKDVHRVHSADEIEREVGK-----
ILQD-PLAHS LN-YADVKRSGEMAATILRHALDAFARLDTTAAVSI LQEDKAL D EBFGRFMRELI TYMME NPRTISVALD LFLIAKAI ERIGDHAKNIAEPIIYVKGTDVHRHSADEIEREVGK-----
LTQAGAQPPLMD-GSALKQAGAMAISMLHRALESFMHLDTAATAQIVRDDCVINEAFHAFVRTLI TYMKE D TQMI STGLDYL SAAKAI ERIGDHATNLAEPYIYVKGTDVHRHSADEIEREVGK-----
LCEE--GSPRGYMETRHIGNQVRVMI HDALDAFARLDAAEQALQVLLADADI DREYQSARTLTYMIE D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
VAGSGI--PRKLA-RGLEHLAELALVQLKEVLDVYASRSPEKANSIRERDEE IDALYTSLFRRELLTYMIE D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
LAEPE--PLKPY-VNINFMSEIVKEMVNDVSI SFIQDQTLAKKVI EKDDTV D ELYHQLEERLMTYVLE D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
LNKEK--HHDIV-KEIWNMGNKVKSMIKDSLDAYVERNLDKAYEVCKRDDVD SLYKRI FNELLANIMSE D KSKVQQLTQPLFVCKYLERIGDRITNVCESTIYLTGTQVLDND-----
LAQEP--PLKRY-VTLPEMGRRLTEMMDALAKAVERDAALARRVLEMDQV D GLYEEVTRRELITYMME D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
LNTMP--PLPQT-ASLRALGQLSAQAVRNSLLSYSNKDEQIATKVRENDARDL DAQYATLFRRELLTYMIE D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
IGESK--VLKPI-PEIPEMGEIVKSMQLQVLYKAYLESDEDAAREIAARDNEV D KLHKIVYQKCNFMQEEPEHEIDVDSQLLVSYQYI ERIGDYVTVNCEWVIVLKSGETIEDLNR-----
LAERP--KLSGL-EGLEQLTGHAVGMARKAIDSFRNGDPSGAREVLDVDRRCNEIERERAGFMEMMRK D GDTVERALHAI FAARSL ERIGDLSLSTNIAESVIFIEEGLNKHSCPPQKV-----
LAQAP--ALKKY-ALSKSRMLSRLEMSQNLRTSLADRVTRAEVTRMDDDEV D ELYEQIQRELVTYMIE D PPRHSRVINVMWVLSL ERIGDHARNIAEQVIYMAKGLDVRHRTKIEIEIEKVVQQRK-----
VKGNV--RVPEIEAALSSEMGNVRVKMVEEMLAAYVKSDDQEARVAAKDAKVGEMVDRINEKGISKMERHPVITGSDTYLNVAATYMLRIGALVTNIGEWIVYLVNTGIEI ELPNPESSNLV-----
VKIND--NYVF-TRLKTMGKLAMLELDLNNAIRNKDPLIKEVIERDEE IDLYVNVIN--TSYLDNDPFPVAGAH--LAARHLERIGDHSNIAESVYVYLTGQHFETFD-----
MKGEE--RIPAVELEINNMGKAVKNMLEALYINGDDEKAYEVAAMDEIV D DYFRDIQKMMVETIQKHPDVAAKVEYFQVLMHL ERIGDYGNICEWIVYLVNTGIEI ELPNPESSNLV-----

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Figure 11. BLAST search alignment of PhoU sequences.

This is an alignment of various PhoU homologues found using a BLAST search. Highly conserved residues are highlighted.

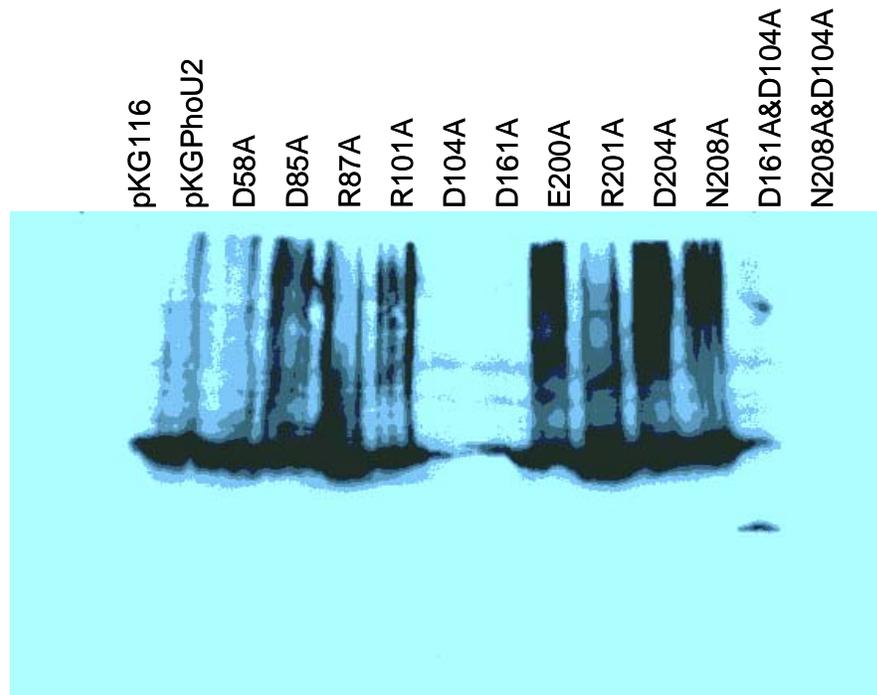


Figure 12. Western blot results.

Results from an immunoblot, blotted using anti-PhoU polyclonal antibodies, of an SDS-PAGE of mutants transformed into BW26337 pRRSCAB are shown in this figure. It is seen that in the control lane of pKG116 there is no PhoU signal. The positive control of pKGPhoU2 shows a strong band. Of the mutants, most show a strong band similar to the positive control. However, D104A, D161A, D161A/D104A, and N208A/D104A lanes showed little or no product. With the D161A/D104A lane there is a smaller band that may be a product of protein degradation.

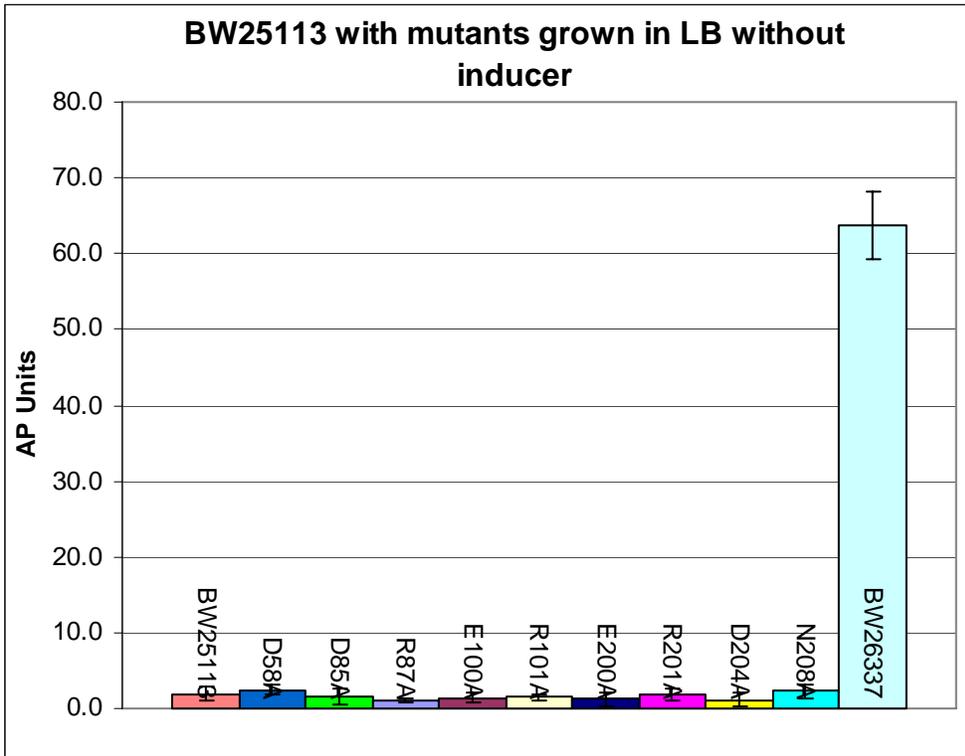


Figure 13. Test for dominance without inducer.

AP assay results of PhoU mutants in the wild-type strain BW25113 grown in LB growth media without any added inducer. It is seen that none of the mutants leads to significant loss of repression of the Pho regulon when compared to the positive control of the BW25113 strain and the negative control of the BW26337 strain.

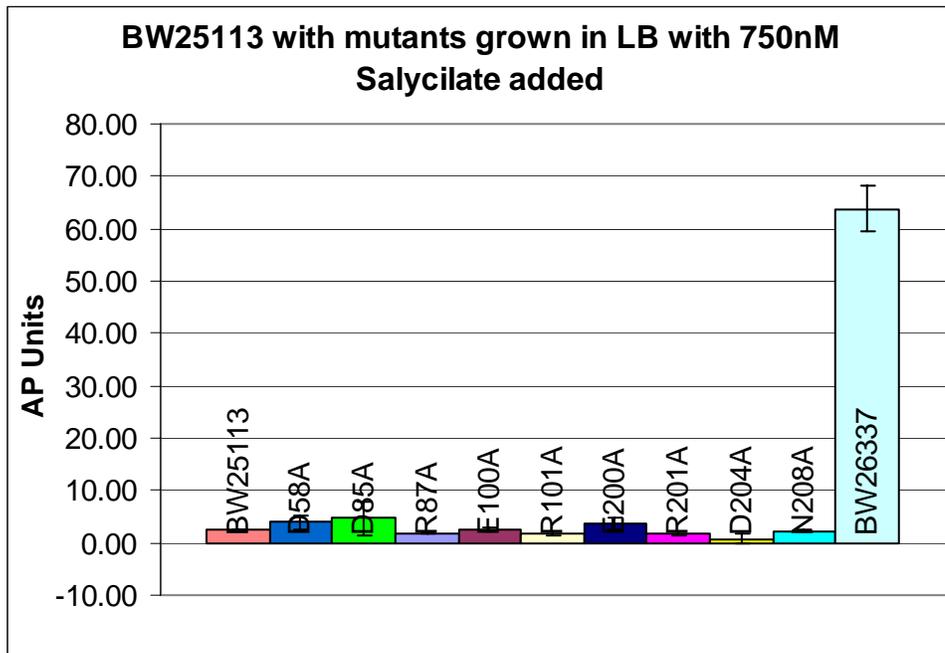


Figure 14. Test for dominance with 750nM inducer.

AP Assay results of PhoU mutants in the wild-type strain BW25113 grown in LB growth media with 750 nM of salicylate. Salicylate is the inducer of the pKG116 derived mutant plasmids. It is seen that none of the mutants leads to significant loss of repression of the Pho regulon when compared to the positive control of the BW25113 strain and the negative control of the BW26337 strain.

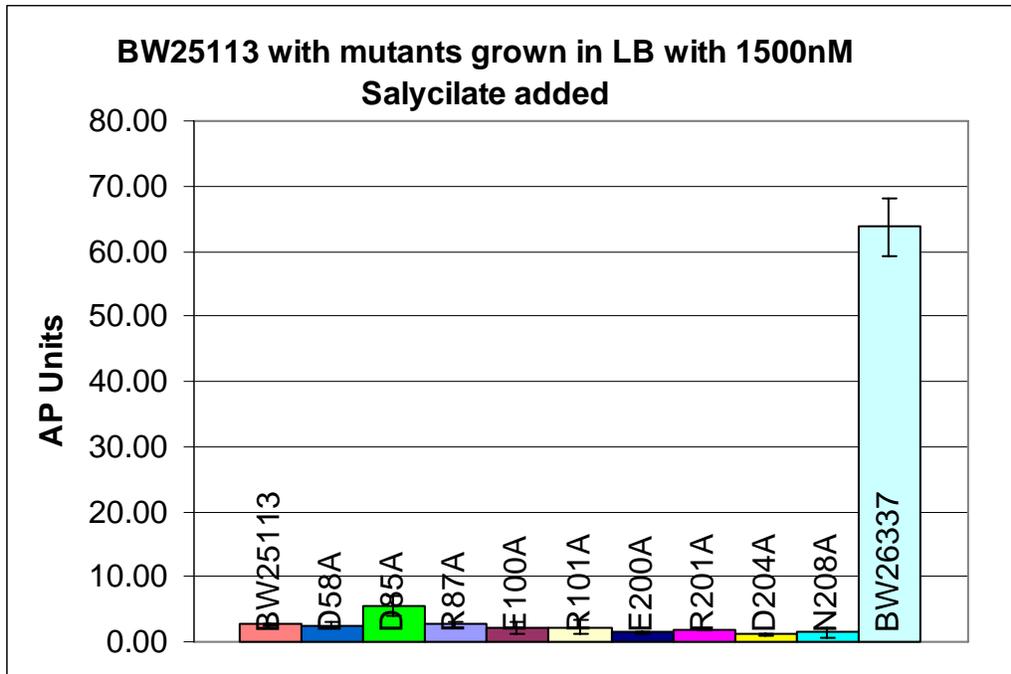


Figure 15. Test for dominance with 1500nM inducer.

AP Assay results of PhoU mutants in the wild-type strain BW25113 grown in LB growth media with 1500 nM salicylate. Salicylate is the inducer of the pKG116 derived mutant plasmids. A t-test performed on the difference between the BW25113 strain and D85A, the highest value of the mutants, resulted in a p-value of 0.2235, which fails an α value of 0.05. It is concluded that none of the mutants leads to significant loss of repression of the Pho regulon when compared to the positive control of the BW25113 strain and the negative control of the BW26337 strain.

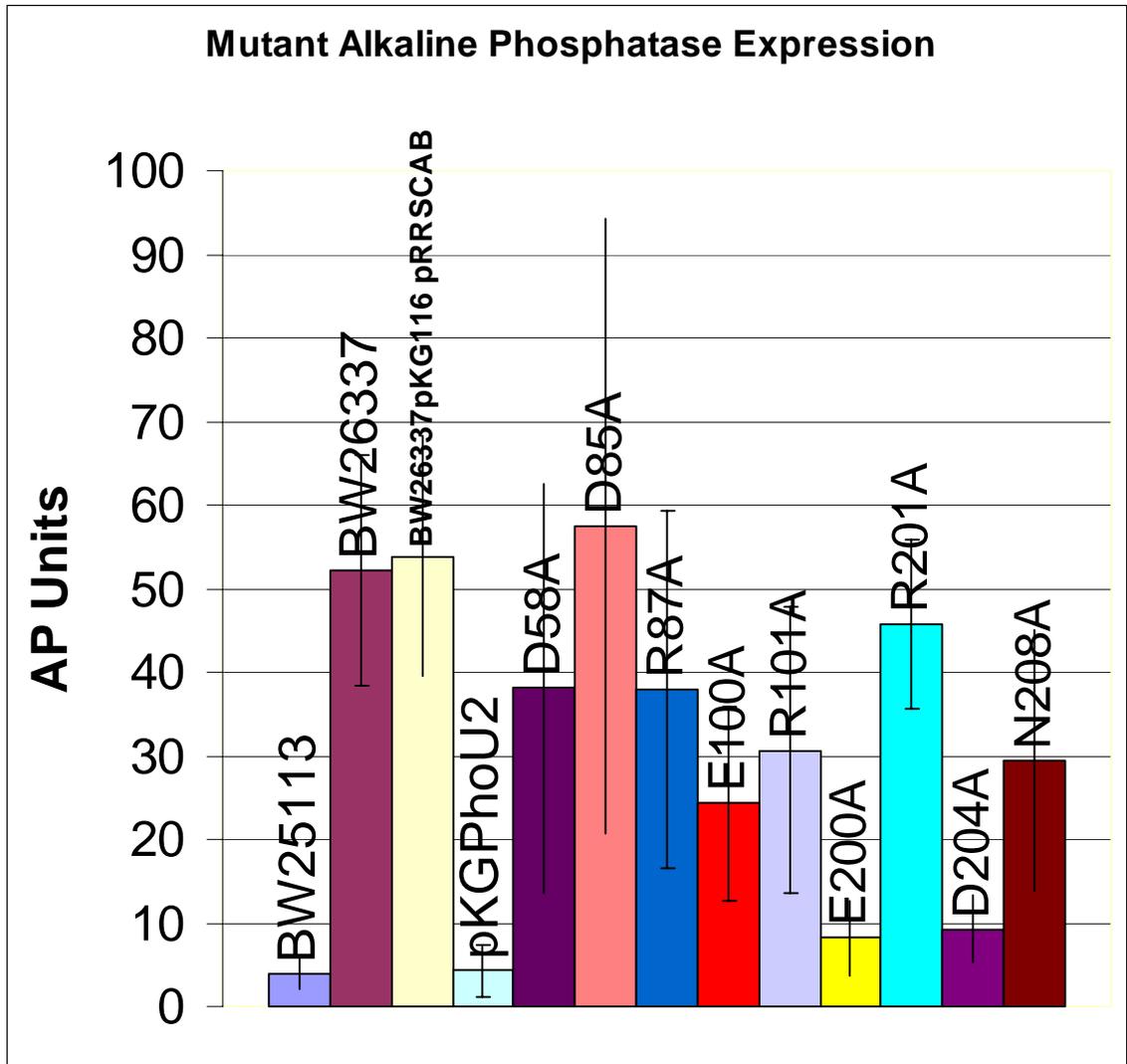


Figure 16. AP Assay results of PhoU mutants.

This graph contains the results of an AP assay performed using BW26337 pRRSCAB containing the different mutant plasmids. The positive controls of the BW25113 wild-type strain and pKGPhoU2 in BW26337 pRRSCAB show repression. The negative controls of the BW26337 strain and pKG116 in BW26337 pRRSCAB show a loss of repression. Of the mutants tested, it is seen that only E200A and D204A appear to maintain repression similar to the positive controls. The other mutants appear to lose some or all repression.