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Employment of a Promoter-Swapping Technique Shows that PhoU Modulates the Activity of the $PstSCAB_2$ ABC Transporter in *Escherichia coli*[▽]

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Expression of the Pho regulon in *Escherichia coli* is induced in response to low levels of environmental phosphate (P_i). Under these conditions, the high-affinity $PstSCAB_2$ protein (i.e., with two PstB proteins) is the primary P_i transporter. Expression from the *pstSCAB-phoU* operon is regulated by the PhoB/PhoR two-component regulatory system. PhoU is a negative regulator of the Pho regulon; however, the mechanism by which PhoU accomplishes this is currently unknown. Genetic studies of *phoU* have proven to be difficult because deletion of the *phoU* gene leads to a severe growth defect and creates strong selection for compensatory mutations resulting in confounding data. To overcome the instability of *phoU* deletions, we employed a promoter-swapping technique that places expression of the *phoBR* two-component system under control of the P_{tac} promoter and the $lacO^{1D}$ regulatory module. This technique may be generally applicable for controlling expression of other chromosomal genes in *E. coli*. Here we utilized P_{phoB}::P_{tac} and P_{pstS}::P_{tac} strains to characterize phenotypes resulting from various Δ*phoU* mutations. Our results indicate that PhoU controls the activity of the $PstSCAB_2$ transporter, as well as its abundance within the cell. In addition, we used the P_{phoB}::P_{tac} Δ*phoU* strain as a platform to begin characterizing new *phoU* mutations in plasmids.

Many signaling pathways that regulate adaptive responses comprise receptors on the cell periphery and signal-processing components and targets in the interior of the cell. In *Escherichia coli*, phosphate (P_i) limitation triggers an adaptive response in which cells acquire P_i with high affinity and utilize alternate phosphorus sources (36). This response is controlled by the coordinated expression of a set of genes known as the Pho regulon. It is thought that the high-affinity P_i-specific transporter $PstSCAB_2$ (i.e., with two PstB proteins) is the receptor for this system. This ABC transporter is composed of the periplasmic P_i binding protein PstS, two integral membrane pore proteins, PstC and PstA, and a peripheral membrane protein, PstB, which binds and hydrolyzes ATP to bring about P_i transport (7, 33). These proteins are encoded in a single operon along with the PhoU protein.

The signaling proteins that operate on the cytoplasmic face of the inner membrane as well as in the cytoplasm are PhoR, PhoB, and PhoU (36). PhoR is a membrane-bound histidine kinase that can serve either as a phospho donor to PhoB or as a phospho-PhoB phosphatase, depending upon its signaling state (5, 14, 16). PhoB is the response regulator of the system, which, when phosphorylated, binds to specific DNA sequences located upstream of Pho regulon genes, called pho boxes, and activates transcription (13, 15, 17). Members of the Pho regulon include alkaline phosphatase (AP), the PhoBR proteins, and the PstSCAB and PhoU proteins. PhoU is a 27-kDa peripheral membrane protein that negatively regulates the signaling pathway (32, 33). The crystal structures of several PhoU homologs have recently been determined (12, 23). They have the same fold consisting of two three-helix bundles that display several conserved metal binding sites consisting of negatively charged patches on one surface of the protein.

We currently think that the $PstSCAB_2$ transporter senses P_i levels and communicates through PhoU with the two-component signaling proteins, PhoR and PhoB. Since PhoB positively controls its own expression by binding to a pho box sequence upstream of its own gene, there is a positive feedback loop that greatly increases the signaling gain of the system (9). Sufficient P_i generates a signal through the $PstSCAB_2$ transporter that represses the Pho regulon by stimulating the phospho-PhoB phosphatase activity of PhoR (5). This phosphatase activity is required because PhoB can be activated when cells are in high-P_i environments through cross talk from the CreC histidine kinase and from the low-molecular-weight phospho donor acetyl phosphate (18, 19, 37, 38). Conversely, when P_i is limiting or when mutations eliminate any component of the $PstSCAB_2$ transporter or PhoU, the Pho regulon becomes fully activated as the autokinase and phospho donor functions of PhoR are stimulated (36). Thus, the default activity of PhoR is autophosphorylation, and transmembrane signal transduction through the $PstSCAB_2$ and PhoU proteins results in stabilization of the phosphatase form of PhoR.

By a mechanism that is not understood, PhoU negatively regulates the Pho regulon. It has been proposed that PhoU interacts with other proteins of the P_i signaling pathway (36), but no such interactions have been demonstrated yet. A simple BLAST search of sequenced genomes revealed that PhoU homologs are abundant in prokaryotes (not shown), which suggests that PhoU plays an important and conserved role in P_i signaling.

To further study the function of the PhoU protein, we created a strain in which the positive feedback loop that controls

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
XL1-Red	*gyrA96 thi-1 supE44 lacZ mutD mutS mutT*::Tn*10* (Tet^r)	Stratagene
ANCH1	Δ*phoBR*::Kan^r	41
BW25113	*lacI*^{q} *rrnB*3 Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	6
BW25141	*lacI*^{q} *rrnB* Δ*lacZ* Δ*phoBR hsdR* Δ*araBAD* Δ*rhaBAD galU endA uidA* (ΔMluI)::*pir*$^+$ *recA1*	6
BM240	MG1655/pKD46 Δ*phoBR*::Kan^r (P1 transduction from ANCH1)	This study
BM241	BM240 Δ*phoU*::Cam^r	
BM248	BW25113/pRR48/pKG116	This study
BM249	BW25113 P_{phoBR}::P_{tac}	This study
BM250	BW25113 P_{phoBR}::P_{tac}/pRR48	This study
BM251	BM250/pKG116	This study
BM252	BM250 Δ*phoU*::frt	This study
BM253	BM252 Δ*phoU*::frt/pKG116	This study
BM255	BM252/p116phoU2	This study
BM261	P_{pstS}::P_{tac} Δ*pitA*::frt Δ*pitB*::frt/pRR48	This study
BM263	P_{pstS}::P_{tac} Δ*pitA*::frt Δ*pitB*::frt Δ*phoU*/pRR48	This study
JW2955-1	Δ*pitB*::Kan^r	3
JW3460-5	Δ*pitA*::Kan^r	3
Plasmids		
pKD3	PCR template plasmid, Cam^r	6
pKD4	PCR template plasmid, Kan^r	6
pPK46	Temperature sensitive, λRed plasmid	6
pCP20	Temperature sensitive, FLP expression plasmid	6
pKE1	PCR template plasmid, Kan^r pKD4 mutated to include EcoRI site	This study
pKE2	PCR template plasmid, Kan^r P_{tac} *lacO*^{ID}	This study
pRR48	pBR322-based replicon, Amp^r *lacI*^{q}	31
pKG116	pACYC184 based, Cam^r *nahR*	4
p116phoU	Cam^r *phoU* expression plasmid; salicylate-inducible promoter complements Δ*phoU* mutation; contains silent G318A and T377C mutations; T377C causes V126A change in PhoU; used only in mutagenesis studies	This study
p116phoU2	Cam^r *phoU* expression plasmid; salicylate-inducible promoter; wild-type *phoU* sequence	This study

phoBR expression was removed and replaced by a P_{tac}, *lacO*^{ID} control module. *phoBR* expression in this strain is independent of external P_i levels and can be controlled by using the gratuitous inducer isopropyl-β-D-thiogalactopyranoside (IPTG). By introducing Δ*phoU* mutations into this strain and another strain in which we swapped out the P_{pstS} promoter, we began to characterize the phenotypes to learn more about the function of PhoU. Based on the results of these experiments, we suggest that in addition to controlling the abundance of the $PstSCAB_2$ transporter through the PhoBR two-component signaling pathway, PhoU also controls its activity.

MATERIALS AND METHODS

Bacterial strains, growth media, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Strains were grown either in LB medium (26) or in morpholinepropanesulfonic acid (MOPS) defined media containing either 0.06% glucose and 2.0 mM P_i (MOPS HiPi medium) or 0.4% glucose and 0.1 mM P_i (MOPS LoPi medium) (22, 36). The Pho regulon was not induced when wild-type cells were grown in LB medium or MOPS HiPi medium and was fully induced when wild-type cells were grown in MOPS LoPi medium. Where indicated, ampicillin, chloramphenicol, and kanamycin were used at concentrations of 100, 34, and 50 μg/ml, respectively. 5-Bromo-4-chloro-3-indolyl phosphate (XPhos) was included in solid media at a concentration of 40 μg/ml.

For growth curve experiments, 0.3-ml portions of overnight cultures grown in MOPS LoPi medium containing ampicillin and chloramphenicol (to avoid accumulation of mutants with compensatory mutations) were used to inoculate 25 ml of prewarmed LB medium containing ampicillin and chloramphenicol to obtain an initial optical density at 600 nm (OD_{600}) of approximately 0.005. The cultures were incubated in baffled 250-ml flasks at 37°C with shaking at 250 rpm, 1-ml samples were removed at the indicated times, and their OD_{600} were determined with a spectrophotometer. When the OD_{600} of undiluted cells was greater than 0.3, cultures were diluted before the values were determined.

For growth yield experiments, single colonies of the indicated strains were picked from freshly streaked plates and were used to inoculate 5-ml portions of liquid media containing appropriate antibiotics. Cultures were grown in glass tubes (16 by 125 mm) at 37°C on a roller drum rotating at 75 rpm. Cell growth as measured by using OD_{600} was determined following 24 h of incubation. Cell cultures grown in LB medium were diluted 1:10 in ice-cold saline before values were determined, whereas cultures grown in MOPS media were not diluted.

Construction of plasmids. To create the template plasmid used for promoter swapping, pKD4 was amplified with the PKE1FOR and PKE1REV primers (Table 2) using inverse PCR. The linear product was digested with EcoRI, ligated with T4 DNA ligase, and transformed into the *pir*$^+$ strain BW25141. The resulting plasmid, designated pKE1, did not contain the ribosome binding site from pKD4 and contained a new unique EcoRI site adjacent to the right FLP recognition site of pKD4 (Fig. 1) (6). To construct pKE2, a short PCR product was obtained by using primers PTACFOR and PTACREV (Table 2) to amplify an 82-bp segment of pRR48 (31) that contained the P_{tac} promoter and the *lacO*^{ID} operator, as well as a ribosome binding site and an initiator ATG codon that was flanked by an EcoRI site and an NdeI site. This PCR product was digested with EcoRI and NdeI and was ligated into similarly digested pKE1 plasmid, creating pKE2.

The p116phoU and p116phoU2 plasmids were constructed by amplifying a chromosomal fragment that contained the *phoU* gene with the PHOUFOR and PHOUREV primers and *Taq* polymerase (Table 2). The resulting PCR fragment was digested with NdeI and KpnI and ligated into similarly digested plasmid pKG116 (4). p116phoU and p116phoU2 were two different isolates obtained from the same transformation. The expression of PhoU from these plasmids was under control of the P_{nahG} promoter and the NahR regulatory protein and was inducible with submicromolar amounts of sodium salicylate. The uninduced levels of PhoU obtained with this plasmid were relatively high (about equal to the level of PhoU in wild-type cells grown in MOPS LoPi medium).

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)[a]
PKE1FOR	CGGAGCGAATTCATATTCATATGG ACCATGGCTAATTCC
PKE1REV	TATCCTCCTTAGTTCGAATTCCGA AGTTCCTATTC
PTACFOR	CGGAGCGAATTCGGCAGTTGACA ATTAATCATCGGC
PTACREV	CAAGCTTGATATCGGATCCTGCAG
PHOUFOR	GCATCACATATGGACAGTCTCAAT CTTAATAAACATATTTCC
PHOUREV	GTCGGTACCTTATTTGTCGCTATC TTTCCCCGCCAGCAG
BTACFOR	CGCCACGGAAATCAATAACCTGA AGATATGTGCGACGAGCGTGTA GGCTGGAGCTGCTTC
BTACREV	GAATTGGAGCTTCATCTTCTACGA CCAGAATACGTCTCGCCATATG ACACTCCTTTAAATTG
DELPHOUFOR	GGTCGTTACGGTTGATTCAGGAGT GCGTTGTGTAGGCTGGAGCTG CTTC
DELPHOUREV	CAAATCCCAATAATTAAGTTATTG GGATTTGTCTGGTGAACATATG AATATCCTCCTTAG
STACFOR	GAATATCAACGCTTATTTAAATCA GACTGAAGACTTTATCTCTGTGT AGGCTGGAGCTGCTTC
STACREV	GTCGCGGCGACAACAGTTGCGAC GGTGGTACGCATAACTTTCATAT GACACTCCTTTAAATTG
DELPHOU2FOR	GGACAGTCTCAATCTTAATAAACA TATTTCCGGCCAGTAACTTCAAG ATCCCCTCACGCTG
DELPHOU2REV	GTCGCTATCTTTCCCCGCCAGCAG TTTATCCAGCTCATCGGAGCGCT TTTGAAGCTGGGG

[a] Pertinent restriction sites are underlined.

Strain construction. To create the strain in which the P_{phoB} promoter was replaced by the P_{tac} promoter, a PCR product was generated from the pKE2 template plasmid using primers BTACFOR and BTACREV (Table 2). The PCR product was purified using a QIAquick (Qiagen) spin column and introduced into BW25113 cells harboring pKD46 by electroporation. The remaining steps used to eliminate the antibiotic resistance cassette were essentially the same as the steps described previously (6).

To create the Δ*phoU* allele in the P_{phoB}::P_{tac} genetic background, the method described above for the promoter-swapping experiments was used, except that we used primers DELPHOUFOR and DELPHOUREV (Table 2) and pKD3 was the template for the PCR. We selected for chloramphenicol resistance, and the recipient strain in the electroporation step was BM240. The resultant strain was designated BM241. The Δ*phoU* mutation was then moved from BM241 into BM249 by P1 transduction (21), and the antibiotic resistance cassette was removed by FLP-mediated site-specific recombination (6).

The P_{pstS}::P_{tac} strain was constructed by using the procedure described above except that we used primers STACFOR and STACREV. Δ*pitA*::Kan^r and Δ*pitA*::Kan^r mutations from the Keio collection (3) were sequentially introduced into this genetic background by P1 transduction and converted to *frt* derivatives using the FLP expression plasmid pCP20 (6). The resulting strain containing pRR48 was designated BM261. A Δ*phoU*::Kan^r mutation was introduced into this strain by amplifying the Kan^r gene from pKD4 using primers DELPHOU2FOR and DELPHOU2REV. These primers generated a PCR product that did not contain the FRT sites normally included in this procedure (6) to prevent homologous recombination between closely spaced FRT sites in the promoter region of the operon and the *phoU* deletion site that would have resulted from normal primer design. The PCR product was purified and then introduced by electroporation into strain BM261 harboring pKD46. Strains were verified by PCR analysis.

Compensatory mutations. Individual colonies of strain BM252 obtained from fresh overnight plates were inoculated into tubes containing 5 ml LB medium containing ampicillin without IPTG. Following 24 h of incubation on a roller drum at 37°C, cells were diluted 1:500 into a second tube containing 5 ml of the same medium and grown for 24 h. Cultures were then diluted 1:10^6, and 200-μl aliquots were spread in triplicate onto LB medium plates containing ampicillin and XPhos. Following overnight incubation, colonies from each plate (usually between 150 and 250 colonies) were counted, and the frequencies of blue and white colonies were determined. Sometimes plates were placed in a refrigerator for 24 to 48 h following overnight incubation so that the blue colony color could deepen before the colonies were counted.

AP and immunoblot assays. AP assays were carried out as described previously (43). Immunoblot assays were performed as described previously (25, 27).

Random mutagenesis. We used the protocol described by the supplier (Stratagene) to mutagenized plasmid p116phoU in the XL1-Red mutator strain. One microliter of the mutagenized plasmid was then used to transform freshly prepared competent cells of strain BM252, which were plated onto LB medium plates containing XPhos, ampicillin, and chloramphenicol. Single blue colonies were purified, and plasmid DNA was isolated and used for a second transformation to confirm that the mutation was plasmid encoded. A colony from the second transformation was then used to inoculate a 5-ml LB medium culture containing 0.4 μM sodium salicylate. After overnight incubation cells were lysed by boiling them in sodium dodecyl sulfate (SDS) loading buffer. The proteins were separated on 12% SDS-polyacrylamide gels and were visualized by Coomassie blue staining. Plasmids from strains that produced full-length protein were then sequenced by the BYU DNA sequencing facility. In the course of our mutagenesis studies we found that while the p116phoU plasmid was fully able to complement a Δ*phoU* mutation, it contained two mutations compared with the wild-type sequence, a T-to-C transition mutation at bp 377 causing a conservative valine-to-alanine change at amino acid 126 and a silent G-to-A transition mutation at nucleotide 318.

P_i transport and accumulation. Cells were grown overnight in 5 ml MOPS LoPi medium containing 0.2 mM IPTG, after which they were washed once with 5 ml MOPS medium that did not contain glucose and P_i. To completely starve the cells of phosphate, the cells were then resuspended to an OD_{600} of ~0.4 in MOPS medium containing 0.4% glucose and 0.2 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 these assays $K_2H^{32}PO_4$ was added to a final concentration of 100 nM (15.9 μCi/ml) to cells at an OD_{600} of 0.01. Samples (100 μl) were removed following 15 and 30 s of incubation, filtered rapidly through 0.2-μm nitrocellulose filters, and washed twice with 5 ml of 10 mM Tris-HCl–0.8% NaCl. Assays were performed in triplicate.

Periplasmic protein preparation. Periplasmic proteins were prepared by using a PeriPreps periplasting kit (Epicentre, Madison, WI) as directed by the supplier,

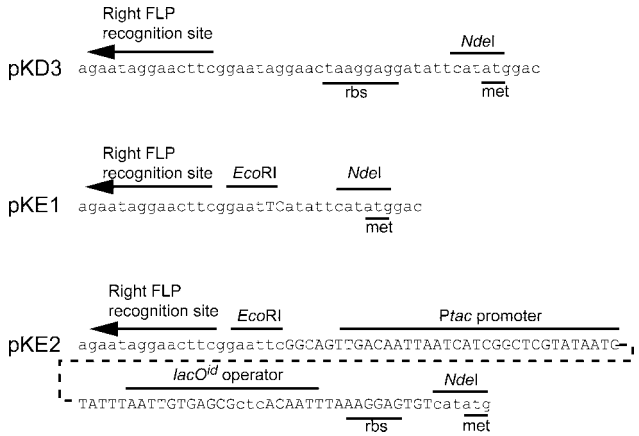

FIG. 1. Steps in construction of the promoter-swapping template vector pKE2. The sequence of the pKD3 parent vector (6) is shown at the top. The sequence of the intermediate plasmid, pKE1, is identical to the sequence of pKD3 except for the differences shown. These differences include introduction of an EcoRI site adjacent to the right FLP recognition site and removal of the original ribosome binding site (rbs). The sequence of the modified region in pKE2 is shown at the bottom. This new plasmid contains the P_{tac}, $lacO^{1D}$, ribosome binding site, and initiator codon region from pRR48 (31). DNA sequence differences between plasmids are indicated by uppercase letters.

except that stationary-phase cells prepared for transport assays were used instead of late-log-phase cells. The proteins were separated on a 10% SDS-polyacrylamide gel and visualized by Coomassie blue staining.

RESULTS

Promoter swapping: creation and characterization of strains. Previous studies have shown that Δ*phoU* mutations, but not Δ*pstSCAB-phoU* mutations, cause a severe growth defect in *E. coli* (8, 30). Both types of mutations lead to full activation of the Pho regulon. Δ*phoU* mutants acquire compensatory mutations in the *pstSCAB* or *phoBR* genes, which relieve the growth defect (30). Compensatory *phoB* mutations inactivate the entire regulon, whereas mutations in the *pstSCAB* genes result in a regulon that is constitutively expressed but has a nonfunctional transporter. These observations suggest that high-level expression of a functional $PstSCAB_2$ transporter in the absence of PhoU is toxic to cells. It has previously been proposed that in addition to its role in signal transduction PhoU may have a metabolic function, perhaps in ATP synthesis (30).

In order to examine the function of PhoU, we modified a strategy employed by Haldimann et al. in their studies of PhoU (8). These workers removed the autogenous regulation of the *phoBR* operon by placing the *phoB* or *phoR* gene under control of the P_{araB} or P_{rhaB} promoter. This was accomplished by integrating a single copy of P_{araB}-*phoB* and P_{araB}-*phoR* or P_{rhaB}-*phoB* and P_{rhaB}-*phoR* gene fusions at the *araCBAD* or *rhaRSBAD* loci of *E. coli* strains lacking *phoB* or *phoR* (8). We also uncoupled *phoB* expression from its usual positive feedback loop, but we replaced the chromosomal P_{phoB} promoter with the P_{tac} promoter and *lac* operator sequence, thereby placing expression of the *phoBR* operon under exogenous control. Our design was different from that of Haldimann et al. in that we kept the *phoBR* genes together as an operon at their normal chromosomal location. This method is also different than the method described by Zhou et al., who integrated single copies of plasmid genes with regulatable promoters into a chromosomal phage attachment site (42). Our promoter-swapping technique was developed by modifying a method used in the Wanner laboratory for creating precise chromosomal deletion mutations (6). We altered the PCR template plasmid, pKD4, so that it contained a P_{tac} promoter, a consensus *lac* operator, a ribosome binding site, and an initiator codon adjacent to the removable antibiotic resistance gene but within the region to be amplified (Fig. 1). Following amplification of this DNA with primers containing terminal homology regions designed so that the *phoB* start codon was replaced by the plasmid start codon, we recombined the PCR product containing the kanamycin resistance gene and the P_{tac} promoter into the chromosome using the λRed system encoded on the pKD46 plasmid (6). The antibiotic resistance marker was then removed by site-specific recombination with the pCP20-encoded FLP recombinase. To decrease basal expression of the *phoBR* genes from the introduced P_{tac} promoter, we introduced into the strain a medium-copy-number plasmid (pRR48) that contains the $lacI^q$ gene. The idea and execution of our method were similar to the idea and execution of the methods described by Alper et al. and by Meynial-Salles et al. (2, 20).

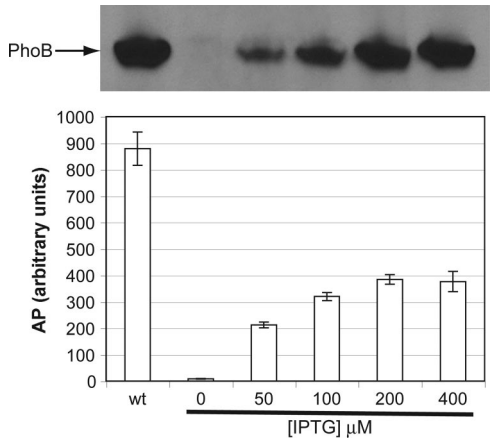

FIG. 2. Expression of PhoB and AP in P_{phoB}::P_{tac} strain BM251. The top panel shows the results of a Western blot analysis of PhoB expression. Equal amounts of cell protein were loaded onto 12% SDS-polyacrylamide gels for immunological detection of PhoB using rabbit polyclonal anti-PhoB sera. The lane on the left contained protein from wild-type BM248 cells (wt) grown in MOPS LoPi medium and shows the amount of PhoU in fully induced cells. The five other lanes contained PhoB from BM251 grown with increasing amounts of IPTG (as indicated below the graph). The graph indicates the AP activity determined for the cells used for Western blotting. Triplicate samples were measured, and the bars and error bars indicate the averages and standard deviations, respectively, of the values obtained.

To investigate the control of PhoB expression in the P_{phoB}::P_{tac} genetic background, we performed an immunoblot assay with strain BM251 grown in a medium that fully induced the Pho regulon in wild-type cells. Cells were grown overnight at 37°C in MOPS LoPi medium containing various amounts of IPTG, and the proteins from harvested cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose, probed with rabbit anti-PhoB sera, and visualized by chemiluminescence. Figure 2 shows that in the absence of inducer, no PhoB was detected, but as the amount of IPTG was increased, the amount of PhoB also increased. There was no difference in the amount of protein between the 200 and 400 μM samples, indicating that the promoter was fully induced with 200 μM IPTG. AP assays were also performed with these cells to examine the Pho signaling pathway. As shown in the lower panel of Fig. 2, the AP levels were low in uninduced cells, but as the IPTG levels were increased, the levels of AP increased. It should be noted that while full induction of the P_{tac} promoter led to high levels of AP, the levels were still only 40 to 50% of the levels observed for wild-type cells grown under identical conditions. In the uninduced state, there still was enough of the PhoB and PhoR proteins for signal transduction to occur, as shown by an eightfold reduction in the level of AP when cells were grown in MOPS HiPi medium (data not shown), but further signal amplification was eliminated. In summary, expression of PhoB in strain BM251 was uncoupled from its normal autogenous control and placed under control of the P_{tac} promoter. We do not know if the larger amounts of PhoB or AP observed with 50 to 200 μM IPTG represented larger amounts of PhoB per cell or higher percentages of cells expressing PhoB at the maximal level. We

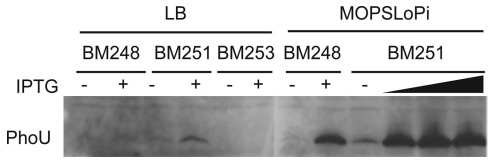

FIG. 3. Expression of PhoU as determined by Western blot analysis. Equal amounts of cell protein were separated by SDS-PAGE, and PhoU was detected with anti-PhoU rabbit polyclonal antiserum. Strains were grown overnight either in the absence or in the presence of 200 μM IPTG in the indicated media. For the BM251 cells grown in MOPS LoPi medium the concentrations of IPTG were (from left to right) 0, 50, 100, and 200 μM.

therefore used 200 μM IPTG for most of the experiments described in this paper.

We also investigated expression of the *pstSCAB-phoU* operon in a wild-type strain (BM248) and in BM251 by performing immunoblotting with antibody raised against PhoU. As shown in Fig. 3, PhoU levels were barely detectable in wild-type cells and in the P_{phoB}::P_{tac} strain grown in LB medium without IPTG. The amount of PhoU increased to a small but detectable level in strain BM251 in LB medium upon addition of IPTG. As a control, no PhoU was detected in Δ*phoU* strain BM253. When BM251 cells were grown in MOPS LoPi medium without IPTG, the amount of PhoU was small, and the level was about the same as the level in the cells grown in LB medium in the presence of IPTG. When the concentration of IPTG in MOPS LoPi medium was increased, the level of PhoU increased significantly above the uninduced levels and quickly reached a plateau with 50 μM IPTG. Increases in expression of *phoU* from the *pstS* promoter occurred with lower IPTG levels than increases in expression of *phoB*, perhaps reflecting the fact that its promoter contained multiple PhoB-binding sites, whereas the *phoB* promoter contains a single pho box (10, 15).

Creation and characterization of a Δ*phoU* strain. Because of the genetic instability of a Δ*phoU* strain and since Δ*phoU* strains accumulate mutations in the *phoBR* operon that relieve the severe growth defect, we initially created a Δ*phoU*::Cam^r mutation in a Δ*phoBR*::Kan^r genetic background and subsequently transferred the *phoU* mutation into the BW250 strain by P1 transduction. The new strain was designated BM252, and when it was incubated on LB medium plates containing XPhos, it formed light blue colonies that were about the same size as the colonies formed by BM250, indicating that the Pho regulon was slightly induced but the severe growth defect was ameliorated. When a compatible plasmid containing *phoU* (p116phoU2) was introduced into the BM252 strain, it formed white colonies on LB medium plates containing XPhos (not shown). Together, these results demonstrated that in the absence of IPTG the Δ*phoU* strain expressed levels of AP that were above the background level (due to activation, but not amplification, of the PhoBR signaling pathway), that this phenotype was due to the *phoU* mutation, and that expression of the wild-type *phoU* gene could complement this phenotype.

Growth curves were then determined for wild-type strain BM248, the P_{phoB}::P_{tac} derivative BM251, its Δ*phoU* derivative BM253, and the Δ*phoU* strain harboring p116phoU2, designated BM255, grown under conditions in which the expression of the Pho regulon was very low (no added IPTG). Approximately equal numbers of cells were transferred from overnight cultures grown in MOPS LoPi medium into LB medium, and growth was measured. As shown in Fig. 4, the strains initially had identical growth rates during the early exponential phase, but then the growth of the Δ*phoU* strain deviated from wild-type growth and the Δ*phoU* strain entered stationary phase at a lower cell density than the other three strains. After 9 h, the OD_{600} of the BM253 strain plateaued at 1.85, which was only 45% of the OD_{600} of the other three strains.

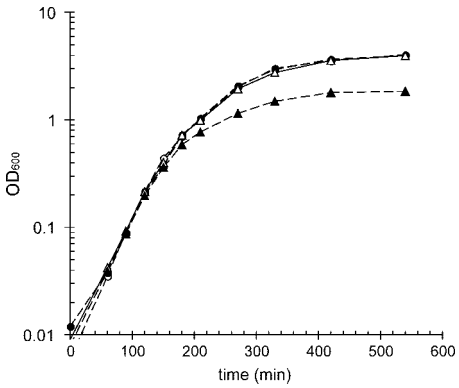

FIG. 4. Growth curves for cells incubated at 37°C in LB medium. Cells were incubated in 25 ml of medium in 250-ml baffled flasks with shaking at 250 rpm. Samples were removed at the indicated times and diluted in phosphate-buffered saline if necessary, and the results were determined with a spectrophotometer at 600 nm. Open circles, BM248; filled circles, BM251; open triangles, BM255; filled triangles, BM253.

To further examine differences in growth, we compared the growth yields of the BM251 and BM253 strains after 24 h of growth in LB, MOPS HiPi, and MOPS LoPi media in the presence or absence of IPTG. As shown in Fig. 5, we observed significant differences in the growth yield between the two strains when they were grown in LB medium regardless of *phoBR* induction and also in MOPS HiPi media, but only with

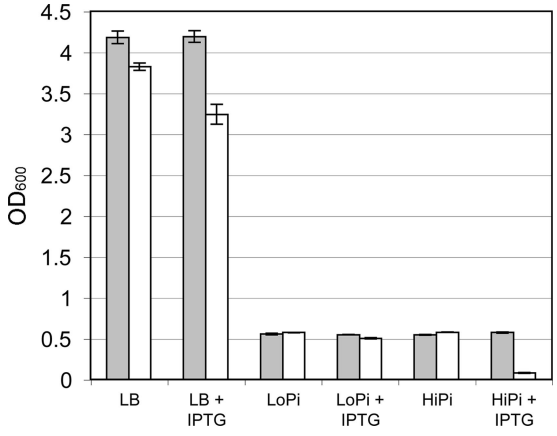

FIG. 5. Growth yields of cells grown at 37°C for 24 h. Gray bars, strain BM251; open bars, strain BM253. Cells were grown in 5-ml cultures in culture tubes (16 by 125 mm) on a roller drum either in the absence or in the presence of 200 μM IPTG in the indicated media, and the OD_{600} were determined. The bars indicate the averages of three trials, and the error bars indicate the standard deviations.

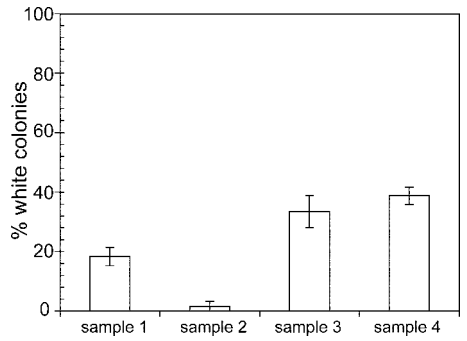

FIG. 6. Compensatory mutations in the Δ*phoU* strain. *E. coli* strain BM252 was grown in LB medium containing ampicillin but no IPTG for 24 h, diluted 500-fold, and then grown for 24 h, after which cells were plated onto LB medium plates containing Xphos. The bars indicate the average percentages of white colonies (presumed *phoB* mutants) in the cultures, and the error bars indicate the standard deviations. The plates contained between 150 and 250 colonies, and three plates were counted for each sample.

phoBR induction. We did not observe a difference in the yields when cells were grown in MOPS LoPi medium under any conditions. Inducing transcription of the *phoBR* operon, and presumably the rest of the Pho regulon, with IPTG magnified the reduction in the growth yield of strain BM253 when cells were grown in LB medium. In MOPS HiPi medium, we observed a reduced growth yield only when the *phoBR* operon was induced by IPTG. Since the concentration of P_i in LB medium was approximately 4 mM (data not shown) and the concentration of P_i in MOPS HiPi medium was 2.0 mM, we observed increasing effects on growth reduction as the phosphate level was increased. In media with the highest levels of phosphate we observed decreases in the growth yield whether the *phoBR* genes were induced or not induced; with intermediate phosphate levels (MOPS HiPi medium) we observed growth reductions only when *phoBR* was induced; and in a low-phosphate medium we never observed a reduction in the growth yield. We concluded that the reduction in growth was a consequence of high external P_i levels.

After we plated overnight cultures of the Δ*phoU* strains BM252 and BM253 growing in LB medium onto LB medium plates containing XPhos, we occasionally observed white colonies, which indicated that there was a reduction in or loss of AP activity. We presumed that the bacteria in these white colonies contained compensatory mutations in the *phoB* gene because when we transformed several of them (arising from BM252) with a plasmid expressing *phoB*, they produced light blue colonies on LB medium plates containing Xphos (not shown), indicating that the mutations had been complemented by *phoB*. We asked how frequently the compensatory mutations arose in cells grown in LB medium in the absence of *phoBR* induction (with no IPTG) following 24 h of incubation and then again following 1:500 dilution into fresh media and a second 24 h of incubation. Four separate 5-ml LB medium cultures of the BM252 strain were grown overnight and plated on LB agar containing XPhos. After 24 h we did not observe any white colonies. However, after dilution and a second overnight incubation, white colonies were obtained for each of the cultures, and the percentage of white colonies in all of the colonies ranged from 0.8 to 19% (Fig. 6). These data are consistent with the appearance of compensatory mutations in the overnight cultures of the Δ*phoU* mutants whose growth yields were reduced, and during subsequent outgrowth the compensatory mutants used the nutrients left in the spent LB medium (probably the remaining amino acids [28]). Since there was such strong selection for growth, any mutation that reduced expression of the $PstSCAB_2$ transporter (including *phoB* mutations) would have accumulated in the cultures. We assumed that other mutations, perhaps mutations in the *pstSCAB* genes, would also accumulate in these overnight cultures, but they would produce blue colonies on the plates and would not be as easy to screen. Perhaps the cultures with low numbers of white colonies actually had accumulated some of these other compensatory mutations. In any event, these results are consistent with a significant reduction in the growth yield of the Δ*phoU* strain, even when the Pho regulon was not induced.

If the PhoU levels in strain BM251 grown in low-P_i media without IPTG (Fig. 3) are an indication of the $PstSCAB_2$ levels in Δ*phoU* strains BM252 or BM253 grown in LB medium without IPTG, then growth defects occur even when $PstSCAB_2$ levels are modest, at best. These growth defects cannot, therefore, be due to runaway expression of *pstSCAB* genes and massive protein accumulation, but rather have some other cause. Since the growth defect was exacerbated in media containing elevated levels of P_i, we think that an elevated intracellular P_i level has a toxic effect despite the low levels of the transporter. Our interpretation of these observations is that they suggest that PhoU may play a role in controlling the activity of the $PstSCAB_2$ protein in high-phosphate environments.

Phosphate transport through the $PstSCAB_2$ protein. To test the hypothesis described above, we employed our promoter-swapping strategy to create a strain in which we could directly control the expression of $PstSCAB_2$ by replacing the P_{pstS} promoter with P_{tac} and then measuring phosphate uptake in isogenic strains whose only difference was the presence or absence of PhoU. To focus on transport through the $PstSCAB_2$ transporter, we needed to eliminate transport through the two low-affinity secondary phosphate transporters, PitA and PitB. We therefore introduced *pitA* and *pitB* deletion mutations into the P_{pstS}::P_{tac} genetic background by P1 transduction. The resulting strains, BM261 and BM263, expressed the $PstSCAB_2$ protein under control of the P_{tac} promoter and utilized this protein as the primary phosphate transporter. Strain BM263 was the Δ*phoU* derivative.

Cells were grown in MOPS LoPi medium containing 200 μM IPTG to ensure that the expression levels of $PstSCAB_2$ were the same and then were starved for phosphate by incubating them for 2 h in MOPS medium containing IPTG but no phosphate. Subsaturating amounts of $^{32}P_i$ (100 nM) were added to the cells, which were collected at various time points, filtered, and washed, and then the amounts of radioactive phosphate incorporated into the cells were determined by scintillation counting. Figure 7 shows that the Δ*phoU* strain BM263 transported phosphate at a ~20% higher rate (2.91 nmol P_i/min/OD_{600} unit) than the $PhoU^+$ strain, BM261 (2.41 nmol P_i/min/OD_{600} unit). When BM261 cells were grown in the absence of IPTG, the rate of phosphate transport was only ~7% of the rate in the presence of

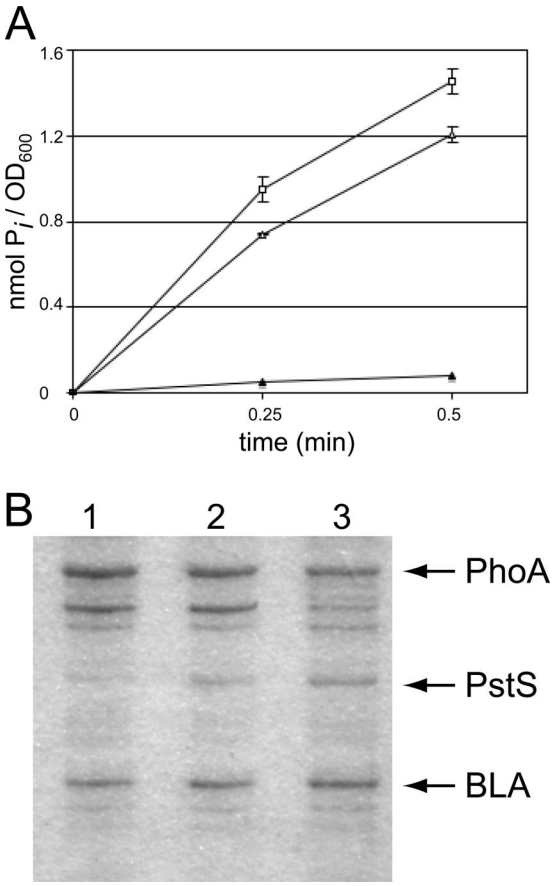

FIG. 7. Phosphate uptake in a Δ*phoU* strain. Strains were grown in MOPS LoPi medium with or without 200 μM IPTG and starved for phosphate. (A) $^{32}P_i$ uptake was determined as described in Materials and Methods. Open squares, BM263 (Δ*phoU*) cells grown in the presence of IPTG; open triangles, BM261 ($PhoU^+$) cells grown with IPTG; filled triangles, BM261 cells grown in the absence of IPTG. The symbols indicate the averages of three separate trials, and the error bars indicate the standard deviations. (B) The periplasmic proteins from the cells used in the transport assays were isolated by osmotic shock, separated by SDS-PAGE on a 10% SDS-polyacrylamide gel, and visualized by Coomassie blue staining. The PhoA, PstS, and β-lactamase (BLA) proteins were tentatively identified based on predicted molecular weights and known subcellular localization. Lane 1, BM261 cells grown in the absence of IPTG; lane 2, BM263 cells grown with IPTG; lane 3, BM261 cells grown with IPTG.

IPTG (0.16 nmol P_i/min/OD_{600} unit), showing that in these experiments phosphate transport was indeed dependent on expression of the $PstSCAB_2$ transporter and not on some other mechanism. So that we could compare transport rates in these strains, we assumed that the levels of expression of the $PstSCAB_2$ transporter under identical culture and growth conditions were equivalent. To test this assumption, we examined the relative amounts of the periplasmic phosphate binding protein PstS. Figure 7B shows a Coomassie blue-stained SDS polyacrylamide gel containing periplasmic proteins isolated from the cells used in the transport assays. Lane 1, which contained proteins from uninduced BM261 cells, has a faint band at the predicted molecular weight of PstS. Lanes 2 and 3, which contained periplasmic proteins from induced BM263 and BM261 cells, respectively, contained larger amounts of PstS. If we normalized the PstS levels for these two strains to the amounts of the β-lactamase enzyme (identified based on its predicted molecular weight), the amounts of PstS were nearly identical. We observed a strong band below the predicted PhoA band in lanes 1 and 2 whose intensity was greatly reduced in lane 3. The identity of the protein in this band is not known, but it may be another binding protein. Other transport experiments (data not shown) showed that the Δ*phoU* derivative BM263 accumulated ~50% more phosphate in 12 min than the BM261 strain. These observations strongly support our hypothesis that PhoU modulates the activity of $PstSCAB_2$ by showing that in the absence of PhoU, cells transport phosphate at a higher rate and accumulate higher levels of phosphate.

Isolation of random mutations in *phoU*. Since we were able to complement a Δ*phoU* mutation with the p116phoU plasmid, we used this plasmid as a target for random mutagenesis to begin studying the structure-function relationships of PhoU. Our strategy was to introduce p116phoU into the XL1-Red mutator strain, which has mutations in three DNA repair pathways that result in a high frequency of mutations. By passaging p116phoU through this strain and then using the plasmid DNA to transform the BM252 strain, we could screen for blue colonies on LB medium plates containing XPhos in which the levels of PhoU activity were decreased. We isolated several blue colonies and verified by using SDS-PAGE that they produced full-length PhoU (not shown). The *phoU* gene from the plasmids was then sequenced to identify the mutations. We found that all of our mutants, as well as our positive control, contained two mutations, G318A and T377C. The T377C mutation caused a valine-to-alanine change at amino acid 126 of PhoU, whereas the G318A mutation was silent. The plasmid DNA that we introduced into the XL1-Red strain must have contained these mutations, even though the plasmid completely complemented the Δ*phoU* mutation. In this background we isolated and preliminarily characterized eight new *phoU* mutations (Table 3). Five of the mutations (P81S, A83T, D85G, K94E, and L99P) clustered in an area of the gene that corresponds to a predicted turn leading to the third helix and in the first half of this 34-amino-acid helix. Two other mutations (C206R and C206Y) involved the same cysteine residue located in the last half of the sixth helix.

To test the PhoU function in these mutants, we measured how well each mutant repressed AP expression in P_i -replete medium (LB broth) containing 200 μM IPTG compared to the expression observed for wild-type plasmid-encoded PhoU (from p116phoU2) (Table 3). Most of the mutations only marginally decreased the ability of PhoU to repress AP activity, although the presence of the L99P mutation consistently resulted in levels of AP that were at least equivalent to the pKG116 control levels, indicating that there was no activity in this mutant. This result is not surprising given that the mutation results in placement of a helix-disrupting residue in the center of a long helix. We also examined the growth yields of the cells carrying the mutant plasmids. For each of the mutants there was a reduction in the growth yield, and there was a rough correlation between the amount of growth reduction and the activity of the PhoU protein in repression of the Pho regulon (Table 3).

TABLE 3. Function of *phoU* mutants

Plasmid or mutation[a]	Signal transduction activity[b]		Growth yield (% of wild-type growth)[c]
	Activity (AP units) (avg ± SD)	% Activity	
pKG116	478.8 ± 28.3	0	67.8
p116phoU	4.9 ± 0.2	100	100
p116phoU2	5.0 ± 0.3	100	100
M26I	32.65 ± 0.4	94.2	91.5
P81S	120.61 ± 4.6	75.6	74.5
A83T	29.78 ± 1.0	94.8	91.1
D85G	73.29 ± 4.6	85.6	86.4
K94E	20.0 ± 0.2	96.8	91.3
L99P	577.3 ± 6.2	−20.8	74.5
C206R	12.3 ± 0.3	98.5	91.7
C206Y	21.12 ± 9.1	96.6	90.0

[a] The designations for the mutations indicate the amino acid in the wild-type PhoU sequence, followed by the residue number and the mutant residue. The mutant PhoU proteins were encoded in the p116phoU plasmid background and contained an additional V126A mutation.

[b] Each of the plasmids was transformed into the BM252 strain, and fresh colonies were grown in 5 ml LB medium containing ampicillin, chloramphenicol, and 200 μM IPTG. The AP activities are the averages ± standard deviations of three separate trials. The percent activity was calculated using the following formula: [(AP activity of pKG116 − AP activity of sample) × 100]/(AP activity of pKG116 − AP activity of p116phoU2).

[c] The growth yield was calculated by culturing the strains overnight to stationary phase and determining the OD_{600} for each culture. The value for a strain encoding wild-type PhoU was determined as follows: (OD_{600} of mutant × 100)/OD_{600} of BM255.

DISCUSSION

We used a promoter-swapping technique to begin a genetic study of a difficult problem in P_i regulation. This method allows precise engineering of strains in which an endogenous promoter is replaced with a P_{tac} promoter, a $lacO^{ID}$ operator, a ribosome binding site, and an initiator codon. This technique should generally be useful to workers who wish to control the expression of chromosomal genes kept as single copies at their original location, perhaps in genetic knockdown or depletion experiments, or to study the regulation of other systems that are autogenously regulated without the confounding variable of changing regulator concentrations. We used this procedure to remove the autogenous regulation of the *phoBR* operon and study the effects of normally unstable Δ*phoU* mutants.

By replacing the P_{phoB} promoter with the P_{tac} promoter we were able to study subdued phenotypes resulting from a Δ*phoU* mutation that in turn provided clues about the function of PhoU. We consistently observed growth defects in Δ*phoU* strains that were manifested by changes in the growth yield, not by changes in the growth rate. It has been suggested previously that PhoU may have a metabolic role, perhaps in ATP synthesis (30). We reasoned that decreased ATP levels would be reflected by lower growth rates, so that function for PhoU seemed unlikely. Cessation of growth in a batch culture usually involves depletion of essential nutrients or accumulation of toxic products. The P_i levels in spent media of a Δ*phoU* strain grown to stationary phase indicated that P_i was still abundant and not limiting (data not shown). This demonstrated that the cessation of growth of a Δ*phoU* strain was not due to increased P_i assimilation and sequestration and supported the suggestion that it may have been due to the accumulation of some inhibitory compound, which could have been intracellular P_i or perhaps some phosphorylated metabolite (8, 30). The intracellular levels of P_i in *E. coli* range from 5 to 20 mM depending on the growth conditions and carbon source, and 10 mM is a common level during growth on glucose (24, 29, 34, 35, 40). Cells appear to possess a P_i homeostasis mechanism that keeps the P_i levels in this range (36). The finding that the growth defect of a Δ*phoU* mutant was exacerbated by increased levels of P_i in the medium is consistent with the proposal that P_i is toxic.

We were surprised to observe a growth defect in the Δ*phoU* P_{phoB}::P_{tac} strain because several lines of evidence suggest that $PstSCAB_2$ expression in this strain is modest at best. We did not have the reagents to directly measure the amount of $PstSCAB_2$, but the amounts of PhoU expressed in low-P_i media in the absence of IPTG should be a good indication of $PstSCAB_2$ levels because the *pstSCAB-phoU* operon is controlled by a single PhoB-dependent promoter (1). We found that the PhoU levels were low to moderate in the uninduced state (Fig. 3). Also, the amount of AP, encoded by another Pho regulon gene, was near the basal level in the uninduced state. Together, these results suggest that the levels of $PstSCAB_2$ in the uninduced state should be modest at best. Therefore, why would moderate expression of the $PstSCAB_2$ transporter lead to reductions in cellular growth yields?

When cells are transferred from low-P_i growth conditions in which the $PstSCAB_2$ transporter is expressed at a high level to high-P_i conditions, the cells adapt readily to their new surroundings, suggesting that it is not just high levels of the $PstSCAB_2$ transporter in the presence of high concentrations of environmental P_i that are toxic to cells but the presence of the $PstSCAB_2$ transporter in the absence of PhoU. We hypothesized that PhoU may also control the activity of the $PstSCAB_2$ transporter. Previous studies conclusively demonstrated that PhoU is not required for P_i transport (30), but its role in $PstSCAB_2$ control has not been investigated yet. We used our promoter-swapping technique to examine P_i transport in a strain in which $PstSCAB_2$ levels could be controlled independent of environmental phosphate. Our data are consistent with the proposal that PhoU inhibits transport by the $PstSCAB_2$ transporter when internal P_i levels are elevated. In this role, PhoU may be an essential protein for regulating phosphate homeostasis. Accordingly, in the absence of PhoU, the $PstSCAB_2$ protein continues to transport P_i even when intracellular P_i levels are high and the cells are poisoned. We suggest that this role for PhoU is in addition to its role in signal transduction, in which it is involved in shifting the biochemical activity of PhoR from an autokinase activity to a phospho-PhoB phosphatase activity. We have not addressed the mechanism of this control, but there are many possibilities, including the following two: (i) PhoU may bind intracellular P_i and then interact with one of the PstSCAB proteins (probably PstB) to inhibit its activity, or (ii) PhoU could be an enzyme that produces a small-molecule inhibitor of $PstSCAB_2$ when intracellular P_i levels are high.

In a recent paper Li and Zhang characterized PhoU as a persistence switch and examined the phenotypes resulting from a Δ*phoU* mutation and a transposon insertion mutation in *phoU* (11). The work of these authors clearly showed that *phoU* mutants were more susceptible to antibiotic treatments and to environmental stresses. Based on our findings, it is possible

that the increased susceptibility that Li and Zhang observed could have been related to the fact that the strains were already stressed by accumulation of a toxic product and were not able to withstand additional insults. Li and Zhang also showed that the cell density in stationary phase of a Δ*phoU* strain was significantly lower than the cell density of a wild-type strain (11). Clearly, further work needs to be performed to measure intracellular P_i levels in mutant strains and the potential accumulation of toxic intermediates.

With the construction of a semistable Δ*phoU* strain, we began to use a genetic approach to study structure-function relationships of PhoU by isolating random mutations in a plasmid version of *phoU*. The mutations with the greatest effects on PhoU function (P81S and L99P) involved either the removal or the introduction of a proline residue. This is not surprising since PhoU consists of six long alpha helices and changes involving proline residues may significantly alter the global protein structure. However, it was somewhat surprising to find that a mutation in the highly conserved D85 residue, which is part of one of the highly conserved metal-binding sites, caused only a minor decrease in PhoU function. In fact, except for the L99P mutation, each of the mutations that we isolated resulted in a protein whose activity was more than 75% of the wild-type activity. Since PhoU is composed of two tandem three-helix bundles, it may be that there is functional redundancy between the two halves of the protein and a functional second site may mask disruptions at only one site.

In our mutants there was a correlation between defects in signal transduction and defects in the growth yield; the greater the residual signaling activity, the greater the growth yield. However, the *phoU* gene was originally characterized by using the *phoU35* allele, which was shown to result from an alanine-to-glutamate change at amino acid 147 (39). This mutation blocked signal transduction but did not affect growth, suggesting that the two functions of PhoU can be genetically separated. Analysis of additional mutants should allow us to better understand the dual functions of PhoU in phosphate metabolism and signaling.

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