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# Use of the *rep* Technique for Allele Replacement To Construct Mutants with Deletions of the *pstSCAB-phoU* Operon: Evidence of a New Role for the PhoU Protein in the Phosphate Regulon

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**The phosphate regulon is negatively regulated by the PstSCAB transporter and PhoU protein by a mechanism that may involve protein-protein interaction(s) between them and the $P_i$ sensor protein, PhoR. In order to study such presumed interaction(s), mutants with defined deletions of the *pstSCAB-phoU* operon were made. This was done by construction of M13 recombinant phage carrying these mutations and by recombination of them onto the chromosome by using a *rep* host (which cannot replicate M13) for allele replacement. These mutants were used to show that Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) mutations abolished $P_i$ uptake by the PstSCAB transporter, as expected, and that Δ*phoU* mutations had no effect on uptake. Unexpectedly, Δ*phoU* mutations had a severe growth defect, and this growth defect was (largely) alleviated by a compensatory mutation in the *pstSCAB* genes or in the *phoBR* operon, whose gene products positively regulate expression of the *pstSCAB-phoU* operon. Because Δ*phoU* mutants that synthesize a functional PstSCAB transporter constitutively grew extremely poorly, the PhoU protein must have a new role, in addition to its role as a negative regulator. A role for the PhoU protein in intracellular $P_i$ metabolism is proposed. Further, our results contradict those of M. Muda, N. N. Rao, and A. Torriani (J. Bacteriol. 174:8057–8064, 1992), who reported that the PhoU protein was required for $P_i$ uptake.**

The phosphate (PHO) regulon in *Escherichia coli* consists of more than 30 genes, all of which probably have a role in the assimilation of different phosphorus (P) sources from the environment. These genes are arranged as eight separate transcriptional units. When the preferred P source ($P_i$) is in excess, it is taken up by the low-affinity $P_i$ transporter (Pit) whose synthesis is not under PHO regulon control. Under these conditions, the PHO regulon is repressed. When the extracellular $P_i$ concentration is less than about 4 μM, the synthesis of the high-affinity $P_i$ transporter (PstSCAB) is induced and $P_i$ is taken up by the PstSCAB transporter. Under these conditions, genes belonging to the PHO regulon such as those for the PstSCAB transporter are induced more than 100-fold (29, 31, 33).

The PHO regulon is controlled by a two-component regulatory system consisting of the PhoB and PhoR proteins. The PhoB protein is the transcriptional activator, and the PhoR protein is the $P_i$ sensor. As such, the PhoR protein has a dual regulatory role. It, therefore, probably exists in two forms: an activator form ($PhoR^A$) and a repressor form ($PhoR^R$). Accordingly, the $PhoR^A$ protein would predominate when $P_i$ is limiting, and the $PhoR^R$ protein would predominate when $P_i$ is in excess (35). Also, the PhoB protein activates transcription only when phosphorylated, and the PhoR protein is a PhoB protein kinase (15). Therefore, the $PhoR^A$ protein is probably a kinase that activates the PhoB protein by phosphorylation, and the $PhoR^R$ protein might act as a protein phosphatase that inactivates the phospho-PhoB protein by dephosphorylation. Accordingly, the transcriptional activation of PHO regulon promoters by the PhoB and $PhoR^A$ proteins during $P_i$ limitation is controlled by the amount of phospho-PhoB protein. In addition, two $P_i$-independent controls activate the PHO regulon. Both the catabolite regulatory sensor CreC and acetyl phosphate activate the PhoB protein by phosphorylation in the absence of the PhoR protein (32, 39).

$P_i$ control of the PHO regulon involves two opposing processes (activation and repression), and these regulate the interconversion of the $PhoR^A$ and $PhoR^R$ proteins. Activation requires no additional factor(s). $P_i$ limitation or a mutation in the *pstSCAB-phoU* operon leads to activation because either leads to formation of the $PhoR^A$ protein. In contrast, repression requires additional factors. An excess of extracellular $P_i$, an intact PstSCAB transporter, and an accessory protein called PhoU are required for repression, because together these lead to formation of the $PhoR^R$ protein (33). Even though $P_i$ repression requires an intact PstSCAB transporter, repression is independent of $P_i$ transport per se (11).

How extracellular $P_i$, the PstSCAB transporter, and the PhoU protein lead to formation of the $PhoR^R$ protein is poorly understood. According to one model, a "repressor complex" containing the PstSCAB transporter, the PhoU protein, and the $PhoR^R$ protein may form when $P_i$ is in excess (31, 33). Hence, protein-protein interactions between the PstSCAB transporter and the PhoR protein or between the PhoU and PhoR proteins may be important for regulating the interconversion of the $PhoR^A$ and $PhoR^R$ proteins. One way to obtain evidence for protein-protein interactions in vivo involves the characterization of suppressor mutations. Isolating a mutation in one gene that suppresses the effect of a mutation in another gene may indicate an interaction between their gene products, especially if the suppression is allele specific.

Although hundreds of mutations in the *pstSCAB-phoU* operon have been identified, only a handful have been well

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characterized. When these genes were sequenced (2, 25), 15 had been defined by complementation. Nine of these were in the *pstS* (formerly called *phoS*) gene, none was in the *pstC* gene, four were in the *pstA* gene (including one *pstA* allele and three former *phoT* alleles), one was in the *pstB* gene, and one was in the *phoU* (also formerly called *phoT*) gene. Some additional mutants were isolated in order to verify the open reading frames inferred from the DNA sequence (25), including two with mutations in the *pstC* (formerly called *phoW*) gene. Numerous *pstSCAB* mutations, including ones made by site-directed mutagenesis, have been subsequently characterized (10, 11, 40). Also, the sole *phoU* mutation (the *phoU35* allele) has been sequenced and shown to be a missense change (A-147→E [9]). Further, many mutants having a lesion in the *pstSCAB-phoU* operon were derived from an *E. coli* K-12 strain called K10, which had been heavily mutagenized (4). Importantly, since K10 is Pit$^-$ and Pst$^-$ Pit$^-$ double mutants cannot transport $P_i$ (and therefore cannot grow on minimal media with $P_i$ as a P source), most K10 *pst* mutants now in common use must have acquired a compensatory mutation(s) in order to grow. Indeed, several have been shown to carry a compensatory mutation that apparently allows for $P_i$ uptake via an alternative anion transporter. Some carry a *glpR* mutation which leads to derepressed synthesis of the GlpT permease for uptake of glycerol-3-phosphate (13). Some have a mutation near the *uhpT* locus for uptake of hexose phosphates, and some have a mutation near, but separable from, the *pstSCAB-phoU* operon itself (data not shown).

In order to avoid complications inherent in the use of strains carrying multiple mutational differences, new mutants carrying defined deletions of the *pstSCAB-phoU* operon were constructed. These were made by the *rep* technique for allele replacement in which an M13 recombinant phage carrying a mutation is used to recombine the allele onto the chromosome by using an *E. coli rep* host, which is unable to replicate M13 (8, 17, 20; also this study). Our studies on these mutants lead us to propose that the PhoU protein has a new role in the PHO regulon, in addition to its role as a negative regulator.

(This work has been described in a thesis submitted by P. M. Steed in partial fulfillment of the requirements for a Ph.D. from Purdue University, May 1992.)

## MATERIALS AND METHODS

**Media, chemicals, and other materials.** Minimal media including MOPS (morpholinepropanesulfonic acid) and M63 and complex media including NZ amine, Luria broth, and tryptone-yeast extract (TYE) were made as described previously (28). Glucose MOPS with 2.0 mM $P_i$ or TYE agar containing an appropriate antibiotic was used for selection of transformants and transductants. Ampicillin was added at 100 or 25 μg per ml, and chloramphenicol was added at 100 or 12.5 μg per ml for plasmid or chromosomal resistance, respectively. Kanamycin was added at 50 μg per ml, spectinomycin and streptomycin were added at 35 μg per ml (each), and tetracycline was added at 15 μg per ml along with 2.5 mM $PP_i$.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (X-P) were obtained from Bachem Fine Chemicals (Torrance, Calif.) and were used at 40 μg per ml for detection of β-galactosidase and bacterial alkaline phosphatase (BAP), respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, Mo.) was added at $3.3 \times 10^{-4}$ M for induction of the *lac* promoter. Low-gelling-temperature agarose was obtained from FMC Corp. (Rockland, Maine).

Restriction endonucleases (New England Biolabs, Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; or Promega Biotec Corp., Madison, Wis.), T4 DNA ligase (Boehringer Mannheim Biochemicals or Promega Biotec Corp.), Klenow fragment of *E. coli* DNA polymerase I and native T7 DNA polymerase (Boehringer Mannheim Biochemicals), and modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Norwalk, Conn.) were used according to the specifications of the manufacturers. [α-$^{32}$P]dATP and carrier-free $^{32}P_i$ were obtained from Amersham Corp. (Arlington Heights, Ill.). The M13 universal primer, 5′-GTAAAAC GACGGCCAGT-3′, and the M13 reverse primer, 5′-ACAG GAAACAGCTATGACC-3′, were obtained from Pharmacia Inc. (Piscataway, N.J.). The mutagenic oligonucleotides were made in the Laboratory for Macromolecular Structure at Purdue University.

**Allele replacement using M13 and a *rep* host.** Plaque-purified lysates of M13 recombinant phage carrying a mutation and an antibiotic resistance gene were prepared, incubated at 65°C for 10 min, and then centrifuged for 3 min to remove residual bacteria. These lysates were used to infect an F′ Δ*lac rep* mutant, such as BW16824, BW17097, or a derivative thereof. Recipient cells were grown in Luria broth to saturation, collected by centrifugation, suspended in 10 mM $MgCl_2$–5 mM $CaCl_2$, and 0.1 ml of cells was infected with 10-μl lysate. The cell-phage mixture was incubated at room temperature for 20 min, and then dilutions were plated onto agar containing an appropriate antibiotic. Several antibiotic-resistant transductants were purified once nonselectively and then streaked onto agar containing 0.3% sodium deoxycholate (sodium DOC). DOC-resistant cells were purified once nonselectively prior to testing for antibiotic resistance and an appropriate mutant phenotype(s). In some cases, medium containing the chromogenic substrate X-P was used to score mutants directly. ColE1 sensitivity was determined by cross-streaking cells against ColE1 colicin (obtained from W. A. Cramer). Agar containing 0.08% sodium DOC was used to select Δ*phoU* mutants, because such mutants were themselves found to be sensitive to higher concentrations of DOC.

**Molecular genetics.** Transductions using P1*kc*, conjugations, and transformations were done as previously described (34). Because P1 grows poorly on a *rep* host, some mutants were made Rep$^+$ before preparation of P1 lysates on them. Strains were made Rep$^+$ by cotransduction with an *ilv* marker using P1 grown on BW6612 (*ilv*::Tn*5*) or BW12598 (*ilv*::Ω) and by selection for kanamycin-resistant (Kan$^r$) or spectinomycin- and streptomycin-resistant (Spc$^r$ and Str$^r$) transductants, respectively. The *rep* and *ilv* mutations are 97% linked by P1 transduction. Transductants were tested for the *rep* allele by testing them for M13 sensitivity. To do this, F′128::Tn*10-11*(*tet*) *lacI*Q1 *lacZ*M15 was introduced into the transductants by patch mating with BW9352 (Table 1) and by selection for tetracycline-resistant (Tc$^r$) exconjugants on glucose-M63 agar containing isoleucine and valine. M13 sensitivity was tested by cross-streaking cells against M13mp18 on TYE agar containing IPTG and X-Gal. Rep$^+$ exconjugants were blue in the region of the cross-streak, and Rep$^-$ ones were white. Because the *pstSCAB-phoU* and *ilv* loci are about 60% linked by P1 transduction, these transductants were also tested for the appropriate *pstSCAB-phoU* mutation.

**Bacteria, plasmids, and phage.** 71.18 was from C. Squires and was routinely used to prepare M13 lysates. CJ236 was from T. A. Kunkel and was used to prepare uracil-containing phage. CU1248 (*ilvY864*::Tn*10*) and Y204 [*ilvG*pΔ(*GMEDAY*)::Ω] were from H. E. Umbarger, and S10 (*ilv*::Tn*5*) was from J. Beckwith. These strains were sources of antibiotic resistance *ilv* markers. JM110 was from J. Messing and was used to prepare

TABLE 1. Bacteria

| Strain[a] | Genotype[b] | Pedigree[c] | Reference or derivation[d] |
|---|---|---|---|
| BW3912 | Δ*lac-169 creB510 thi* | XPh1a, BW1589 | 28 |
| BW4366 | Δ*lac-169 zaj-39*::Tn*10 phoF465 rpsL267 creB510 aroB crp-72 thi* | XPh1a, BW4557 (28) | Tc$^r$ with P1 on P2472 |
| BW6111 | Δ*lac-169 pstS21 ilv-691*::Tn*10 creB510 thi* | XPh1a, BW3912 | Tc$^r$ with P1 on BW6044 |
| BW6504 | Δ*lac-169 phoU35 creB510 thi* | XPh1a, BW5023 | 28 |
| BW6612 | Δ*lac-169 ilv*::Tn*5 creB510 rpsL267 thi* | XPh1a, BW3908 (19) | Kan$^r$ with P1 on S10 |
| BW6833 | Δ*lac-169 pstS21 creB510 thi* | XPh1a, BW6111 | Ilv$^+$ with P1 on BW4366 |
| BW7311 | F′pOX38::Tn*10-11*(*tet*)/Δ*lac-169* IN(*rrnD-rrnE*)*1 ara tsx*(?) *trpA9605 his-29 malT ilv-1 trpR* | FE103, BW391 | 41 |
| BW7873 | *phoB23 phoU35 rpsL267 creB510 thi* | XPh1a, BW7785 | Pro$^+$ with P1 on HL12 (36) |
| BW9352 | F′128::Tn*10-11*(*tet*) *pro*(*BA*)$^+$ *lacI*Q1 *lacZ*M15/Δ*lac-169 proC* IN(*rrnD-rrnE*)*1 rpsL ilv-1 his-29 trpA9605 trpR tsx ara malT* | FE103, BW979 | 34 |
| BW12598 | DE3(*lac*)X74 *ilv*GpΔ(*GMEDAY*)::Ω *arcA** | MG1655$_{MC}$, BW11331 (34) | Spc$^r$ Str$^r$ with P1 on Y204 |
| BW13711 | DE3(*lac*)X74 | BD792, BW13635 | 18 |
| BW14087 | DE3(*lac*)X74 *phoB23* | BD792, BW13989 | Pro$^+$ with P1 on BW7873 |
| BW16824 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 Δ*phoA532 rep-71 phn*(EcoB) | BD792, BW16816 | 20 |
| BW16947 | λRZ5*lacP-phoU*$^+$(PS15) F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 Δ*phoA532 rep-71 phn*(EcoB) | BD792, BW16824 | Amp$^r$ with λRZ5(PS15) |
| BW17024 | M13ps37Δ λRZ5*lacP-phoU*$^+$(PS15) F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 Δ*phoA532 rep-71 phn*(EcoB) | BD792, BW16947 | Kan$^r$ with M13ps37, then Doc$^r$ and ColE1$^s$ |
| BW17041 | M13ps37Δ DE3(*lac*)X74 | BD792, BW13711 | Kan$^r$ with P1 on BW17024 |
| BW17096 | DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW16805 | Pro$^+$ with P1 on BW13711 |
| BW17097 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17096 | Tc$^r$ exconjugant with BW7311 |
| BW17127 | M13ps37 integrant F′pOX38::Tn*10-11*(tet)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17097 | Kan$^r$ with M13ps37 |
| BW17138 | DE3(*lac*)74 Δ*phoU559*::*kan* | BD792, BW17041 | Spontaneous blue on X-P agar |
| BW17142 | DE3(*lac*)74 Δ*phoU599*::*kan* | BD792, BW13711 | Kan$^r$ with P1 on BW17138 |
| BW17146 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71* Δ*phoU559*::*kan phn*(EcoB) | BD792, BW17128 | Doc$^r$ |
| BW17152 | DE3(*lac*)X74 *phoB23* Δ*phoU559*::*kan* | BD792, BW14087 | Kan$^r$ with P1 on BW17138 |
| BW17261 | M13ps44 integrant F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)74 *rep-71 phn*(EcoB) | BD792, BW17097 | Kan$^r$ with M13ps44 |
| BW17265 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71* Δ(*pstSCAB-phoU*)*560*::*kan phn*(EcoB) | BD792, BW17097 | Doc$^r$ |
| BW17335 | DE3(*lac*)74 Δ(*pstSCAB-phoU*)*560*::*kan* | BD792, BW13711 | Kan$^r$ with P1 on BW17280 |
| BW17448 | DE3(*lac*)74 *phoB23* Δ(*pstSCAB-phoU*)*560*::*kan* | BD792, BW14087 | Kan$^r$ with P1 on BW17333 BW17604 |
| BW17604 | M13ps46 integrant F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17097 | Kan$^r$ with M13ps46 |
| BW17610 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 Δ(*pstB-phoU*)*571*::*kan rep-71 phn*(EcoB) | BD792, BW17606[e] | Doc$^r$ |
| BW17617 | M13ps51 integrant F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17097 | Spc$^r$ Str$^r$ with M13ps51 |
| BW17623 | M13ps52 integrant F′pOX38::Tn*10-11*(tet)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17097 | Str$^r$ Str$^r$ with M13ps52 |
| BW17624 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71* Δ(*pstSCAB-phoU*)*560*::Ω *phn*(EcoB) | BD792, BW17623 | Doc$^r$ |
| BW17670 | F′pOX38::Tn*10-11*(*tet*)/DE3(lac)X74 Δ*phoU559*::Ω *rep-71 phn*(EcoB) | BD792, BW17617 | Doc$^r$ |
| BW18018 | M13ps55 integrant F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 *rep-71 phn*(EcoB) | BD792, BW17097 | Kan$^r$ with M13ps55 |
| BW18020 | F′pOX38::Tn*10-11*(*tet*)/DE3(*lac*)X74 Δ*phoU559 rep-71 phn*(EcoB) | BD792, BW18018 | Doc$^r$ |

[a] All strains are *E. coli* K-12 derivatives, except that the *phn*(EcoB) locus is from *E. coli* B.

[b] The *pstS21* allele (formerly called *phoS21*) originated from C86 (27). The *arcA** allele is a leaky *arcA* mutation. M13ps37Δ is an apparent deletion mutant of M13ps37 (see the text). ?, allele uncertain.

[c] Ancestor strain from another lab and its immediate ancestor in this lab.

[d] DOC$^r$, DOC resistant; ColE1$^s$, ColE1 colicin sensitive.

[e] BW17604-like.

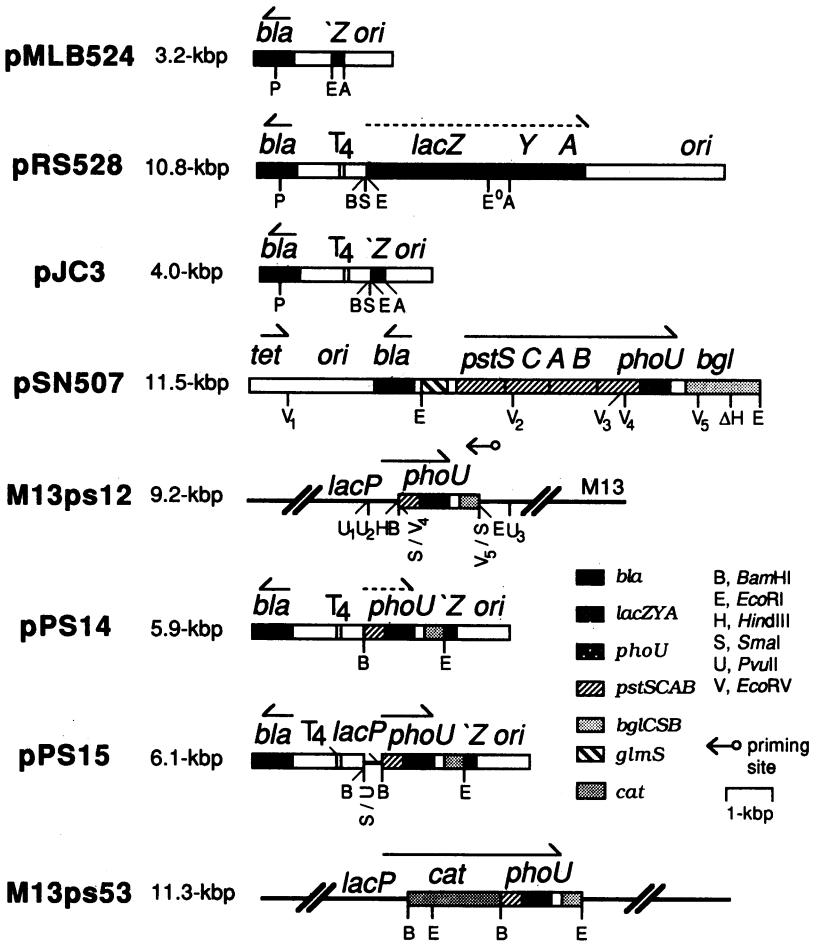


FIG. 1. Structures of plasmids. pMLB524 (6), pRS528 (24), and pSN507 (3) are described elsewhere. The construction of pJC3, M13ps12, pPS14, pPS15, and M13ps53 is described in Materials and Methods. Junctions verified by DNA sequencing are indicated by arrows with a circle at one end. Four tandem copies of the *rrnB* transcription terminator are indicated ($T_4$). Only relevant restriction sites are shown. $E^0$, an *Eco*RI site eliminated by mutation; ΔH, a *Hin*dIII deletion internal to the *bglCSB* operon.

M13 replicative-form DNA for digestion with *Bcl*I. MC1061 was from M. Casadaban and was used for routine plasmid transformation. P2472 (*zaj-39*::Tn*10*) was from P. Reeves. Other bacteria are described in Table 1.

Plasmids and phages are described in Fig. 1 to 3 or below. M13mp19 was from J. Messing (16), and pRS528 was from R. Simons (24). Ones described previously included λRZ5 (1), M13mp18 (18), pHP45Ω (18), pMLB524 (30), pSKS101 (20), pSKS114 (41), and pSN507 (28). pJC3 was made in a separate study involving regulation of the *creABCD* operon. In that study, a 6.1-kbp *Eco*RI fragment carrying a *creAB-lacZ* fusion made with Mu dl (38) was subcloned into pMLB524, to make a $LacZ^+$ plasmid (called pDY7). pDY7 was digested with *Hin*dIII and *Sal*I, filled in with Klenow fragment, and religated to construct a $LacZ^+$ *creA-lacZ* fusion plasmid (pJC1). The 2.5-kbp *Pst*I-to-*Eco*RI fragment of pJC1 (which originated from pMLB524) was ligated to the 1.5-kbp *Pst*I-to-*Eco*RI fragment of pRS528 to make the $LacZ^-$ plasmid pJC3 (Fig. 1) (12).

M13ps11 and M13ps12 (Fig. 1) are similar except for the orientation of the *phoU*$^+$ gene. M13ps11 was made by ligation of the 1.9-kbp *Eco*$RV_4$-to-*Eco*$RV_5$ fragment containing the *phoU*$^+$ gene in pSN507 to *Sma*I-cut M13mp18. To do this, pSN507 was also digested with *Bst*EII to destroy a comigrating 1.9-kbp *Eco*RV fragment. M13ps12 was made by ligation of the *Eco*RI-to-*Bam*HI fragment containing the *phoU*$^+$ gene in M13ps11 to *Eco*RI- and *Bam*HI-cut M13mp19. pPS14 was made by ligation of the 1.9-kbp *Bam*HI-to-*Eco*RI fragment containing the *phoU*$^+$ gene from M13ps12 to *Bam*HI- and *Eco*RI-cut pJC3. pPS15 was made by ligation of the 2.0-kbp *Pvu*$II_2$-to-*Eco*RI fragment containing the *phoU*$^+$ gene together with the *lacP* promoter from M13ps12 to *Sma*I- and *Eco*RI-cut pJC3. Consequently, the *phoU*$^+$ gene in pPS14 is expressed at a low basal level because of the absence of a promoter, while the *phoU*$^+$ gene in pPS15 is expressed from the *lacP* promoter. M13ps53 was made by ligation of the 2.1-kbp *Bam*HI *cat* cassette from pSKS114 (23) to *Bam*HI-cut M13ps12.

M13 phages carrying deletions of the *pstSCAB-phoU* operon (see Fig. 3) were made as follows. M13ps21 was made by replacing the 1.4-kbp *Eco*RI fragment in M13ps19 (Fig. 2) with the *Eco*RI *kan* cassette from pSKS101. M13ps22 was made by ligation of the 0.3-kbp *Bcl*$I_3$-to-*Bcl*$_4$ fragment in M13ps20 (Fig. 2) to *Bam*HI-cut M13mp19. M13ps23 was made by ligation of the 2.4-kbp *Sph*I-to-*Sma*I fragment of M13ps21 to *Sph*I- and *Hin*cII-cut M13ps22. M13ps37 was made by ligation of the 2.4-kbp *Xba*I fragment of M13ps23 to *Sma*I-cut M13ps22, after the ends were filled in with the Klenow fragment. M13ps47 was

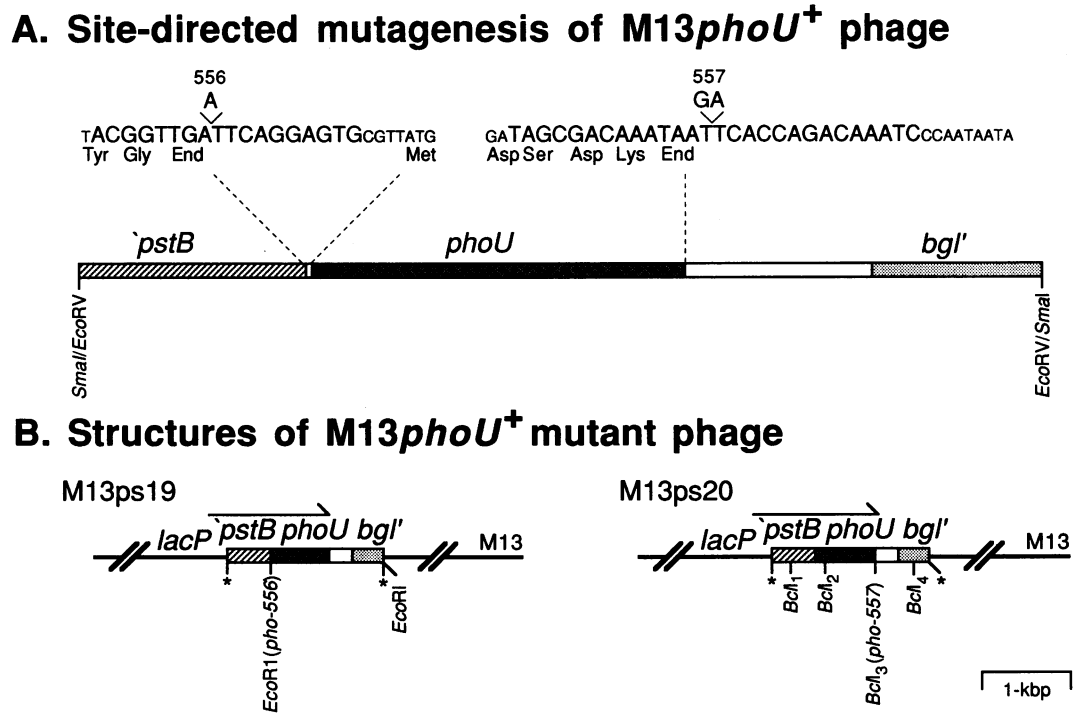


FIG. 2. Construction of M13*phoU*$^+$ phage with flanking restriction sites. (A) An *Eco*RI site was introduced immediately upstream of the *phoU*$^+$ gene in M13ps12, within the *pstB*-to-*phoU* intergenic region, by using the complement of the oligonucleotide shown in large font on the left. A *Bcl*I site was introduced immediately downstream of the *phoU*$^+$ gene in M13ps12 by using the complement of the oligonucleotide shown in large font on the right. The *Eco*RI and *Bcl*I sites were designated *pho-556* and *pho-557*, respectively. Bases inserted are shown above. (B) M13ps19 and M13ps20 carrying the *pho-556* and *pho-557* alleles are shown. Only relevant restriction sites are indicated. Asterisks, sites destroyed by the construction of M13ps12 (Fig. 1).

made by ligation of the 5.3-kbp *Eco*RI-to-*Bsp*HI fragment containing the *bgl* sequences in M13ps22 to the 2.8-kbp *Eco*RI-to-*Bsp*HI fragment of M13ps37. M13ps55 was made by ligation of the *Bam*HI *kan* cassette from pSKS101 to *Bam*HI-cut M13ps47. M13ps51 was made by insertion of the *Sma*I Ω cassette containing the *aadA* gene from pHP45Ω into *Sma*I-cut M13ps47. M13ps43 was made by ligation of the 0.3-kbp *Pst*I-to-*Dra*I fragment extending from within the 3′ end of the *glmS* gene to a few bases upstream of the *pstS* promoter in pSN507 to *Pst*I- and *Sma*I-cut M13mp18. M13ps44 was made by ligation of the 7.2-kbp *Eco*RI-to-*Bsp*HI fragment containing the *kan* and *bgl* sequences in M13ps37 to the 2.5-kbp *Eco*RI-to-*Bsp*HI fragment of M13ps43. M13ps48 was made by ligation of the 7.3-kbp *Eco*RI-to-*Bsp*HI fragment containing the *bgl* sequences in M13ps44 to the 2.8-kbp *Eco*RI-to-*Bsp*HI fragment of M13ps37. M13ps52 was made by ligation of the *Sma*I Ω cassette from pHP45Ω into *Sma*I-cut M13ps48. M13ps45 was made by ligation of the 2.7-kbp *Hpa*I fragment extending from within the *pstS* gene to the intergenic region between the *pstA* and *pstB* genes in pSN507 to *Sma*I-cut M13mp18. M13ps54 was made by digestion of M13ps45 with *Pvu*I followed by religation. M13ps46 was made by ligation of the 7.3-kbp *Eco*RI-to-*Bsp*HI fragment containing the *kan* and *bgl* sequences in M13ps37 to the 2.5-kbp *Eco*RI-to-*Bsp*HI fragment of M13ps54. In some cases, DNAs were partially digested with an enzyme, and the appropriate fragments were gel purified.

**Construction of λ*phoU*$^+$ phage.** pJC3 has a polylinker with *Bam*HI, *Sma*I, and *Eco*RI sites flanked by *lac* and *bla* sequences in common with and in the same orientation as λRZ5. Therefore, an insert in the pJC3 polylinker can be recombined onto λRZ5 by homologous recombination. Also, such λRZ5 recombinant phages are selectable as Amp$^r$ transductants. The *phoU*$^+$ gene was recombined from pPS14 or pPS15 onto λRZ5 to make λRZ5*phoU*$^+$(PS14) or λRZ5*lacP-phoU*$^+$ (PS15), respectively. λRZ5*phoU*$^+$ (PS14) expresses the *phoU*$^+$ gene at a low level; it complements *phoU* mutants on some media but not on other media. Apparently, a weak promoter in this phage allows for low-level expression of the *phoU*$^+$ gene, which is sufficient for complementation on some media. λRZ5*lacP-phoU*$^+$(PS15) expresses the *phoU*$^+$ gene from the *lacP* promoter; it complements *phoU* mutants on all media (data not shown).

DNA fragments in the pJC3 polylinker were recombined onto λRZ5 as follows. A BW13711 transformant containing the recombinant plasmid was grown in NZ broth with 0.4% maltose for about 4 h. Cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$, and 0.1 ml of cells was infected with 1 μl of a λRZ5 lysate (about $10^6$ phage). After 20 min of absorption, 2.5 ml of NZ broth was added, and the mixture was incubated at 37°C for 2 to 4 h until lysis. BW13711 was infected with these lysates, and lysogens containing λRZ5 recombinant phage were selected on TYE agar with 25 μg of ampicillin per ml. These lysogens were UV irradiated, and the resultant phage were plaque purified in order to prepare new lysates. These lysates were used to make new lysogens by streaking phage on a lawn of recipient bacteria and by purifying cells from plaque centers on TYE agar containing 25 μg of ampicillin per ml. These lysogens were confirmed by their immunity to λ*cI*h80 and used in complementation studies.

**Molecular biology techniques.** The isolation and manipulation of plasmid, chromosomal, and M13 replicative-form

DNAs and DNA sequencing by the dideoxy chain termination method using modified T7 DNA polymerase were done as described previously (18, 34). DNA fragments were gel purified by using low-gelling-temperature agarose as described previously (19). Oligonucleotide-directed mutagenesis using native T7 DNA polymerase (5) was done as described elsewhere (14). DNA transfer and hybridization using GeneScreen Plus (du Pont de Nemours & Co., Boston, Mass.) were carried out according to the manufacturer's instructions. Chromosomal DNAs were isolated from the M13 integrants, deletion recombinants, and a wild-type strain. These DNAs were digested with restriction endonucleases, separated by electrophoresis using 0.7% agarose gels, blotted onto two membranes, and probed with randomly labeled probes as described previously (34).

**Measurement of $P_i$ uptake.** Cells were grown to the stationary phase, collected by centrifugation, washed twice with 0.4% glucose–MOPS without $P_i$, resuspended at an optical density at 660 nM of approximately 0.05, and incubated at 37°C for 2 h. $^{32}P_i$ was added at 2 μM (final concentration) to 2.5 ml of cells, and 0.5-ml portions were transferred at 5-s intervals onto 0.45-μm-pore-size nitrocellulose membranes and washed twice with 0.5 ml of 0.85% saline by using a Millipore model 2702550 sampling manifold (Millipore Corp., Bedford, Mass.). Membranes were dried, and the radioactivity was measured with a Beckman model LS7000 liquid scintillation counter (Beckman Scientific Instruments, Irvine, Calif.). Samples containing known amounts of $^{32}P$ were used to calculate the $P_i$ concentration.

**Enzyme assays.** BAP was assayed in $CHCl_3$- and sodium dodecyl sulfate-treated cells, and cell debris was removed before the absorption was determined for activity measurements as done previously (37). Units are nanomoles of product made per minute at 37°C and were calculated by using the molar extinction coefficient $E_{410}$ of $1.62 \times 10^4$ for *p*-nitrophenol.

## RESULTS

**Construction of deletions of the *pstSCAB-phoU* operon.** Three kinds of deletions were made. One removed only the *phoU* gene, another encompassed the entire *pstSCAB-phoU* operon, and a third deleted the adjacent *pstB* and *phoU* genes. The Δ*phoU* mutation was made by introduction of an *Eco*RI site (the *pho-556* allele) immediately 5′ and a *Bcl*I site (the *pho-557* allele) immediately 3′ to the *phoU* gene and by deletion of intervening DNA. These sites were introduced into separate M13*phoU*$^+$ phage by site-directed mutagenesis (Fig. 2), and these phages were used to construct M13 recombinant phage carrying Δ*phoU* mutations (Fig. 3). M13ps37 carries the Δ*phoU559*::*kan* mutation in which the *kan* gene is substituted for the *phoU*$^+$ gene. M13ps55 carries the Δ*phoU559* mutation and, in addition, a *kan* gene in the M13 polylinker. M13ps51 carries the Δ*phoU559*::Ω mutation in which the Ω fragment with the *aadA* gene is substituted for the *phoU*$^+$ gene. These Δ*phoU* mutations were assigned the same allele (Δ*phoU559*) because the same DNA segment is deleted in each. Also, the antibiotic resistance genes in the Δ*phoU559*::*kan* and Δ*phoU559*::Ω mutants are in the same orientation as the *pstSCAB-phoU* operon. In this orientation, expression of the resistance gene is unlikely to interfere with expression of the upstream *pstSCAB* genes. The orientation of the *kan* gene in M13ps55 is unimportant, because in this case, the antibiotic resistance gene is lost upon recombination of the Δ*phoU559* mutation onto the chromosome.

M13 phage carrying two Δ(*pstSCAB-phoU*) mutations were made. M13ps44 carries the Δ(*pstSCAB-phoU*)*560*::*kan* mutation, and M13ps52 carries the Δ(*pstSCAB-phoU*)*560*::Ω mutation (Fig. 3). These deletions are similar except for the antibiotic resistance marker. Both extend from a *Dra*I site a few bases upstream of the *pstS* promoter to the *Bcl*I site (the *pho-557* allele) immediately 3′ to the *phoU* gene. The antibiotic resistance genes in these alleles are in the same orientation as the upstream *glmS* gene so as not to interfere with *glmS* gene expression. In addition, a phage carrying the Δ(*pstB-phoU*)*571*::*kan* mutation, M13ps46, was made. This deletion extends from the *Hpa*I site in the *pstA*-to-*pstB* intergenic region to the *Bcl*I site introduced 3′ to the *phoU* gene. As in the other deletion-substitution mutants, the *kan* gene is in the same orientation as the upstream *pstSCA* genes. These alleles were recombined onto the chromosome as described below.

**Allele replacement of *pstSCAB-phoU* mutations using the *rep* technique.** Mutations were recombined onto the chromosome by a two-step procedure for allele replacement. First, integrants containing an M13 recombinant phage integrated into the chromosome were isolated by infection of an *E. coli rep* mutant and by selection of antibiotic-resistant transductants. Second, recombinants lacking M13 sequences were selected on agar containing DOC. Because a *rep* mutant is blocked in replication of single-stranded phage such as M13 (26), M13 recombinant phage are maintained in a *rep* mutant by integration into chromosomal DNA via one homologous recombination event (8, 17, 20; also, this study). These integrants were selectable as antibiotic-resistant transductants because an antibiotic resistance gene had been cloned into the M13, which could be in any nonessential region of the phage. Also, *rep* mutants with a Δ*lac* mutation were used to prevent integration into the chromosome via homologous *lac* sequences carried by common M13 vectors. Further, these *rep* mutants carried F′pOX38::Tn*10-11*(*tet*), so M13 could be introduced by infection. Because cells carrying M13 are DOC sensitive (7), recombinants that had lost M13 sequences by a subsequent crossover event were selectable as DOC-resistant cells.

Eventually, all six Δ(*pstSCAB-phoU*), Δ(*pstB-phoU*), and Δ*phoU* mutations were recombined onto the chromosome by using the respective M13 recombinant phage and a *rep* host, as described in Materials and Methods. In these constructions, 10 antibiotic-resistant transductants were routinely purified, and most of these gave rise to recombinants, among which nearly half had the expected antibiotic resistance and mutant phenotypes. As expected, these Δ(*pstSCAB-phoU*), Δ(*pstB-phoU*), and Δ*phoU* mutants synthesized BAP constitutively. However, when this method was first used to recombine the Δ*phoU559*::*kan* allele onto the chromosome, no BAP-constitutive mutant was isolated. Hence, it was considered that the Δ*phoU* mutation may be deleterious.

Allele replacement of the Δ*phoU559*::*kan* mutation was, therefore, carried out by using a *rep* mutant (BW16947) that is diploid for the *phoU*$^+$ gene. In this strain, one *phoU*$^+$ gene is at the *pstSCAB-phoU* locus and another is on a λ phage, at the *att*λ site. Accordingly, BW16947 was infected with M13ps37 (Δ*phoU559*::*kan*), Kan$^r$ transductants were selected, and DOC-resistant derivatives of these were selected and tested for cells that were Kan$^r$ and ColE1 sensitive. ColE1 sensitivity was tested to identify those recombinants that had lost M13 sequences because cells containing M13 are ColE1 insensitive (7). P1 was grown on several Kan$^r$, ColE1-sensitive derivatives and used to infect a *rep*$^+$ strain (BW13711), and Kan$^r$ transductants were selected. Although these transductants were not themselves BAP constitutive, they readily segregated BAP-constitutive derivatives upon purification on BAP indicator agar (in the absence of DOC). Apparently, the transductants

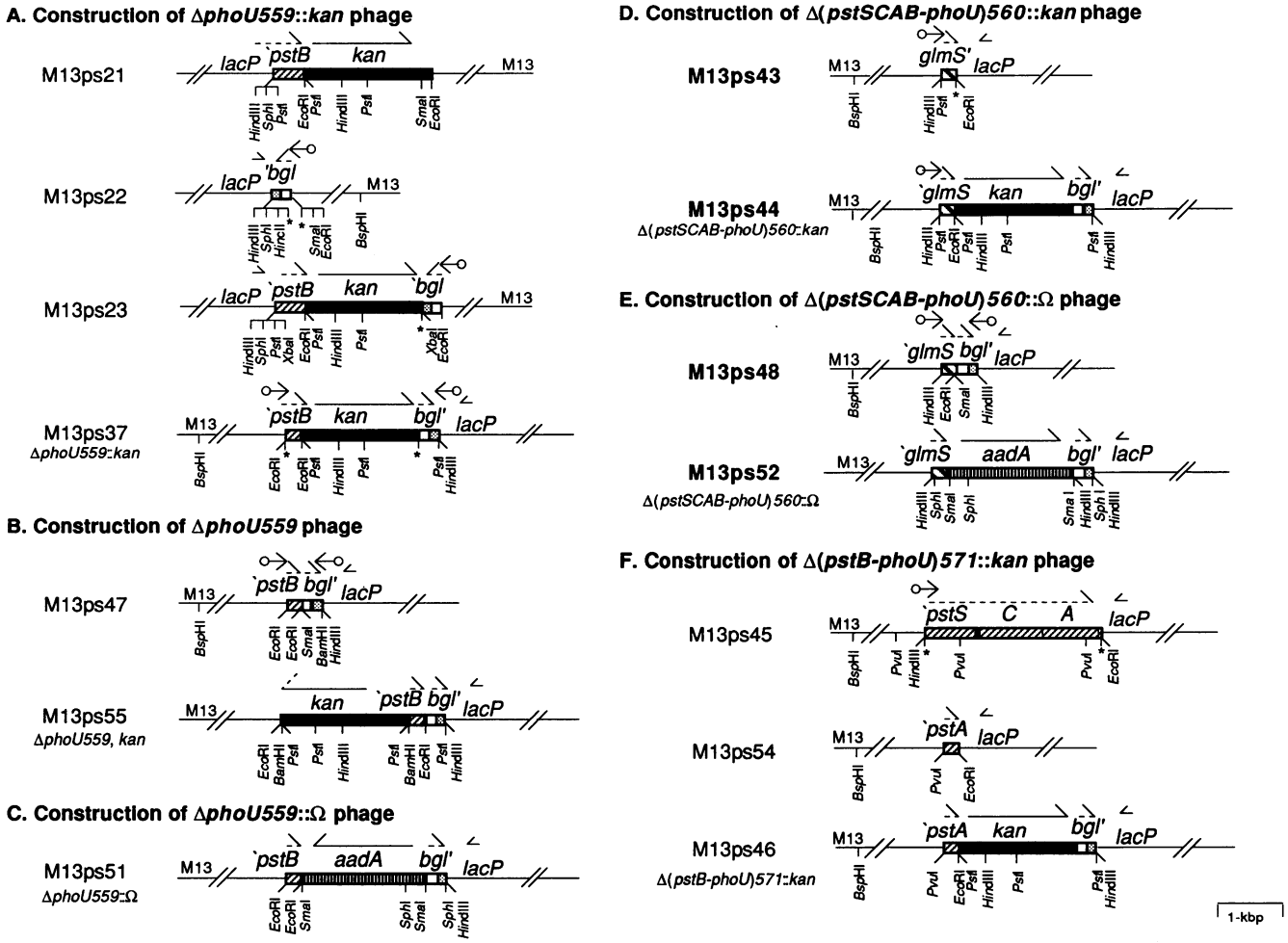


FIG. 3. Construction of M13 recombinant phage carrying deletions of the *pstSCAB-phoU* operon. Junctions verified by DNA sequencing are indicated by arrows with a circle at one end. Solid arrows, orientation of transcription for the *aadA* and *kan* genes and the *lacP* promoter; dashed arrows, orientation of the *glmS*, *pst*, and *bgl* gene segments; open rectangles, the region between the 3′ end of the *phoU* gene and the 5′ end of the *bglCSB* operon. Only relevant restriction sites are shown.

themselves were not constitutive, because they carried both the *phoU*$^+$ gene and the Δ*phoU559*::*kan* mutation at the *pstSCAB-phoU* locus. Such transductants (e.g., BW17041; Table 1) might carry the Δ*phoU559*::*kan* mutation on an M13 derivative (M13ps37Δ) that is DOC resistant and ColE1 sensitive. Such a diploid strain would be expected to segregate *BAP*-constitutive Δ*phoU* mutants (such as BW17138; Table 1) by homologous recombination. Anyway, it was later shown that these segregants had the structure expected of Δ*phoU* mutants. They were also shown to be more DOC sensitive than wild-type cells. Subsequently, all Δ*phoU* mutations were recombined onto the chromosome by using a modified two-step procedure in which DOC-resistant recombinants were selected on agar containing less DOC, as described in Materials and Methods.

Unexpectedly, the Δ*phoU559*::*kan* mutants grew extremely poorly. It was considered that this growth defect might not be caused by the Δ*phoU* mutation but rather be due to overexpression of the *kan* gene. Because the *kan* gene in these mutants is immediately downstream of the *pstSCAB* genes, the *kan* gene is expected to be expressed at a very high level from the strong *pstS* promoter because of derepression of the PHO regulon. To rule out this possibility, two additional Δ*phoU* mutations were constructed. One (Δ*phoU559*) has a simple deletion containing no antibiotic resistance marker; the other (Δ*phoU559*::Ω) has the Ω fragment containing the *aadA* gene flanked by transcription terminators (22) substituted for the *phoU* gene. Because mutants carrying these Δ*phoU* mutations also grew extremely poorly, the growth defect is a consequence of the Δ*phoU* mutation. This was further substantiated as described below. In contrast, Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) mutants did not show the severe growth defect characteristic of Δ*phoU* mutants. It was, therefore, especially important to verify that these mutants had other properties expected of *pstSCAB* and *phoU* mutations.

**Verification of *pstSCAB-phoU* mutants.** All Δ(*pstSCAB-phoU*), Δ(*pstB-phoU*), and Δ*phoU* mutations showed the expected linkage to the *ilv* locus in P1 crosses (data not shown). Also, these mutations were transferred by P1 transduction into a λ$^-$ F$^-$ Δ*lac* mutant (BW13711) that is otherwise wild type. These transductants were shown to be complemented by pSN507, which carries the entire *pstSCAB-phoU* operon. In addition, the Δ*phoU* transductants were shown to be complemented by λRZ5*lacP-phoU*$^+$(PS15), while the Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) transductants were shown not to be

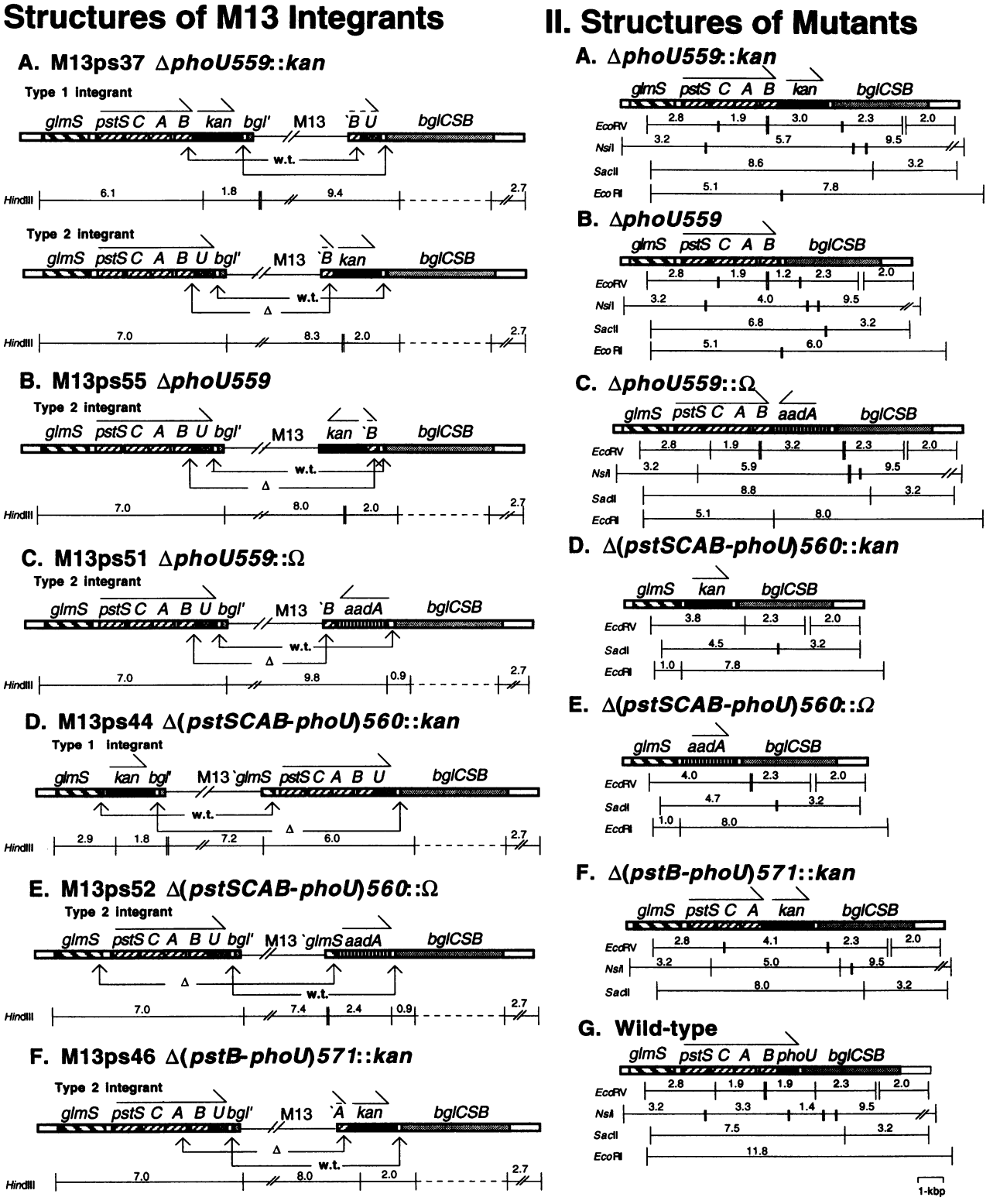


FIG. 4. Construction of Δ*phoU*, Δ(*pstSCAB-phoU*), and Δ(*pstB-phoU*) mutants. (I) BW17097 [F'pOX38::Tn*10-11*(*tet*)/Δ*lac*X74 *rep*] was infected with the indicated M13 recombinant phage, and antibiotic-resistant transductants were selected. Integrants formed by a crossover event upstream of the deletion are designated type 1, and those formed by a crossover event downstream of the deletion are designated type 2. Crossover events leading to the formation of wild-type (w.t.) or deletion (Δ) recombinants are indicated. (II) DOC-resistant recombinants were selected from integrants whose structures are shown in panel I. Structures were verified by DNA hybridization (Fig. 5). The bottom line(s) in each section shows the arrangement of restriction fragments for enzymes used to verify the structures. Half-barbed arrows indicate orientation of transcription. The dashed segment shows a *Hin*dIII fragment absent from probes used for DNA hybridization.

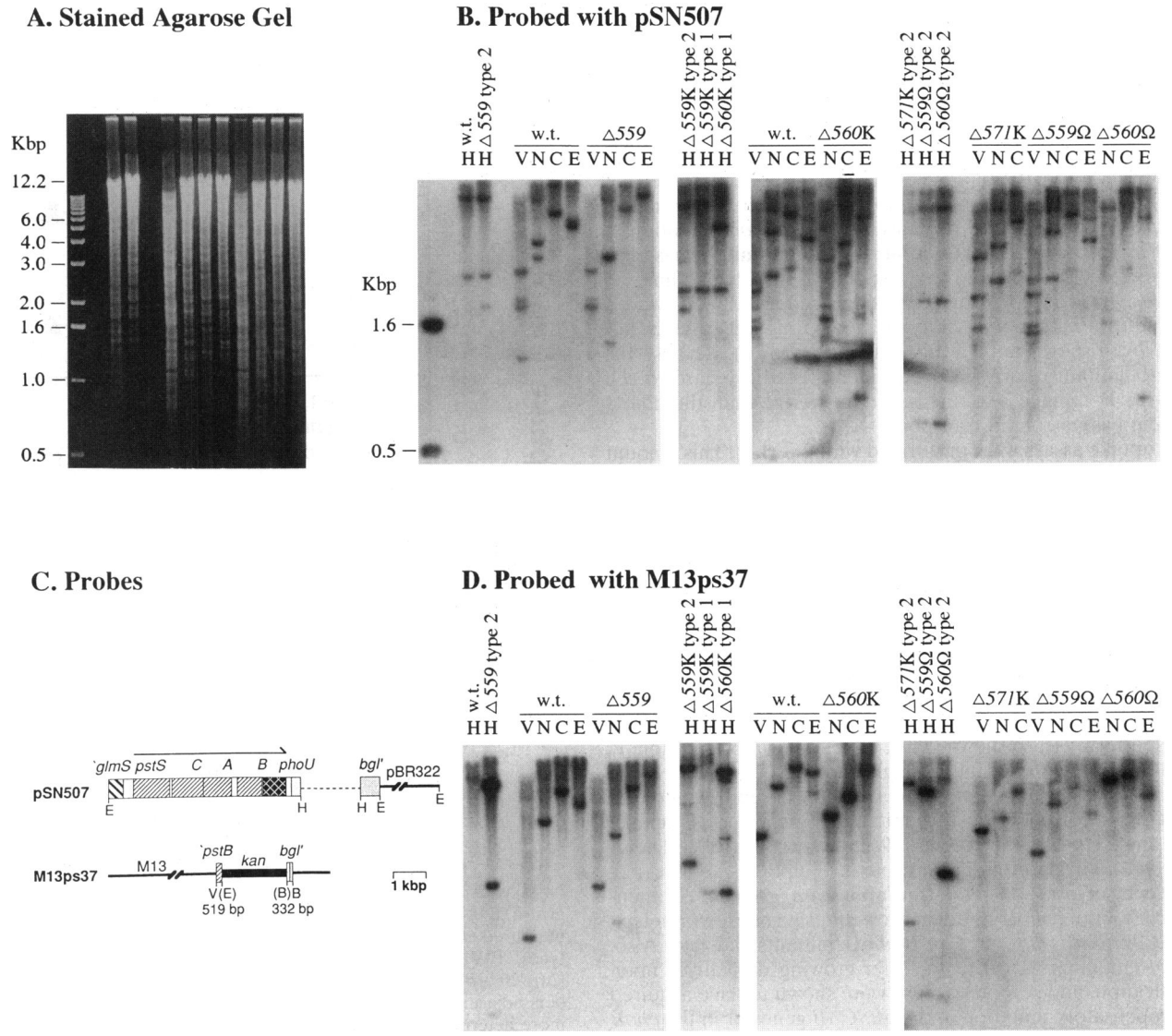


FIG. 5. Hybridization of chromosomal DNAs. (A) One stained agarose gel. (B) A composite autoradiograph of membranes probed with pSN507. (C) pSN507 contains a part of the *glmS* gene, the entire *pstSCAB-phoU* operon, and *bgl* sequences on an 8.2-kbp *Eco*RI fragment. It is a derivative of pBR322 containing an 11.8-kbp *Eco*RI insert from which a 3.6-kbp *Hin*dIII fragment internal to the *bglCSB* operon was deleted (3). M13ps37 has 519 bp of *pstB* DNA upstream of the *Eco*RI site, E, and 340 bp downstream of the *Bcl*I site, B, which were introduced by site-directed mutagenesis. *Eco*RV, V, and *Bam*HI, H, sites from an M13 vector are shown. (D) A composite autoradiograph of membranes probed with M13ps37. Strains examined included BW13711 (wild-type [w.t.]), BW17138 (Δ*phoU559*::*kan* [Δ559K]), BW17127 (Δ559K type 1), BW17128 (BW17127-like; Δ559K type 2), BW18020 (Δ*phoU559* [Δ559]), BW18018 (Δ559 type 2), BW17670 (Δ*phoU559*::Ω [Δ559Ω]), BW17617 (Δ559Ω type 2), BW17265 [Δ(*pstSCAB-phoU*)*560*::*kan* (Δ560K)], BW17261 (Δ560K type 1), BW17624 [Δ(*pstSCAB-phoU*)*560*::Ω (Δ560Ω)], BW17623 (Δ560Ω type 2), BW17610 [Δ(*pstB-phoU*)*571*::*kan* (Δ571K)], and BW17604 (Δ571K type 2). B, *Bam*HI; C, *Sac*II; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; V, *Eco*RV.

complemented by this phage, as expected. The Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) transductants were shown to be complemented with regard to regulation of BAP synthesis. The Δ*phoU* transductants were shown to be complemented with regard to both regulation of BAP synthesis and the severe growth defect (data not shown).

The physical structure of the *pstSCAB-phoU* region was verified by DNA hybridization for both the integrants and their respective deletion recombinants. Integration of the M13 recombinant phage was expected to form two types of integrants. These integrants are distinguishable by digestion with *Hin*dIII (Fig. 4I), while the deletion mutants are distinguishable by digestion with multiple diagnostic enzymes (Fig. 4II). In some cases, integration of the phage was expected to cause a BAP-constitutive phenotype because of interruption of the *pstSCAB-phoU* operon by the M13. The results from representative DNA hybridization experiments are shown in Fig. 5. In brief, these results demonstrated that both types of integrants were formed and that their physical structures always corresponded to the pattern of restriction fragments predicted on the basis of their BAP phenotypes. Also, all deletion mutants had the pattern of restriction fragments predicted. It was also shown with pSN507 and M13ps37 as probes that M13 sequences were present in the integrants and absent in the deletion mutants.

In addition, the Δ*phoU559*::*kan* mutation was rescuable by

an M13*phoU*$^+$ phage. To do this, BW17146 (F'pOX38::Tn*10*-11(*tet*)/Δ*phoU559*::*kan rep*) was infected with M13ps53 (*phoU*$^+$ cat; Fig. 1), integrants were selected as Cm$^r$ transductants, and recombinants lacking M13 sequences were selected as DOC-resistant cells. Only two kinds of chloramphenicol-sensitive recombinants were obtained: ones that were Kan$^r$ and BAP constitutive and grew poorly (like BW17146) and ones that were Kan$^s$ and BAP repressed and grew well (as expected for wild-type recombinants). Therefore, the severe growth defect of Δ*phoU* mutants must be caused by the Δ*phoU* mutation.

**Effects of *pstSCAB-phoU* mutations on $P_i$ uptake.** It has been previously shown that *pst* mutations abolished high-affinity $P_i$ uptake while a *phoU* mutation had no effect (25). However, that study used a mutant carrying the *phoU35* allele, which has since been shown to be a missense mutation (9). Because the Δ*phoU* mutant clearly behaved differently with regard to cell growth, $P_i$ uptake was measured to verify that the PhoU protein has no role in transport.

$P_i$ uptake assays were performed with 2 μM $P_i$. This amount of $P_i$ is sufficient for saturation of the PstSCAB transporter ($K_t$, 0.2 μM), yet it is low enough to avoid substantial (greater than 16%) uptake by the Pit transporter ($K_t$, 10 μM). The results for control strains in Fig. 6A show that $P_i$ uptake was substantially decreased by the *pstS21* allele while the *phoU35* allele was without effect. Similar data have been reported previously (25). The results in Fig. 6B show that $P_i$ uptake was dramatically decreased by the Δ(*pstSCAB-phoU*)*560* and Δ(*pstB-phoU*)*571* mutations, as expected. Both of these mutations reduced uptake about 30-fold (Fig. 6B and data not shown). That $P_i$ uptake under these conditions was due to the PstSCAB transporter was also shown by the effect of a *phoB* mutation. $P_i$ uptake was decreased about eightfold by the *phoB23* allele (Fig. 6B), because this mutation abolishes derepression of the *pstSCAB-phoU* operon by $P_i$ limitation. In contrast, $P_i$ uptake was unaffected by the Δ*phoU559* mutation (Fig. 6B). Therefore, the PhoU protein has no role in $P_i$ uptake.

**Effects of *pstSCAB-phoU* mutations on growth.** As mentioned above, Δ*phoU* mutants showed a severe growth defect, while *pstSCAB-phoU* and *pstB-phoU* mutants did not. Also, Δ*phoU* mutants gave rise to faster-growing derivatives upon purification, and several of these were shown to have acquired a compensatory mutation in the *pstSCAB* genes or in the *phoB* or *phoR* gene (data not shown). Therefore, the effects of known mutations in these genes were tested.

The effect of a Δ*phoU* mutation on growth can be seen in Fig. 7. Growth of the Δ*phoU* mutant was barely detectable following a 24-h incubation, while growth was poor after 36 h. In contrast, both the wild-type and the Δ(*pstSCAB-phoU*) strains grew well after 24 h. Also, *phoB* strains including a *phoB* Δ*phoU* mutant grew well after 24 h.

It is well known that mutants derepressed for the PHO regulon generally grow less well than otherwise isogenic wild-type strains. This slowed growth is probably a physiological consequence due to derepression of such a large number of genes (39). Importantly, this cannot explain why a severe growth defect is specific to a Δ*phoU* mutant, because Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) mutants are also fully derepressed for the PHO regulon. Also, this growth defect is apparently not caused by the type of medium. A severe growth defect due to a Δ*phoU* mutation was seen on MOPS or M63 minimal media containing a variety of carbon sources, on complex media, and on complex media containing glucose (data not shown). It should also be pointed out that faster-growing mutants with a compensatory mutation also rapidly accumulated during growth of Δ*phoU* mutants in broth cultures. Hence, it was necessary to repurify and routinely test Δ*phoU* mutants in order to avoid cultures containing large numbers of compensatory mutants.

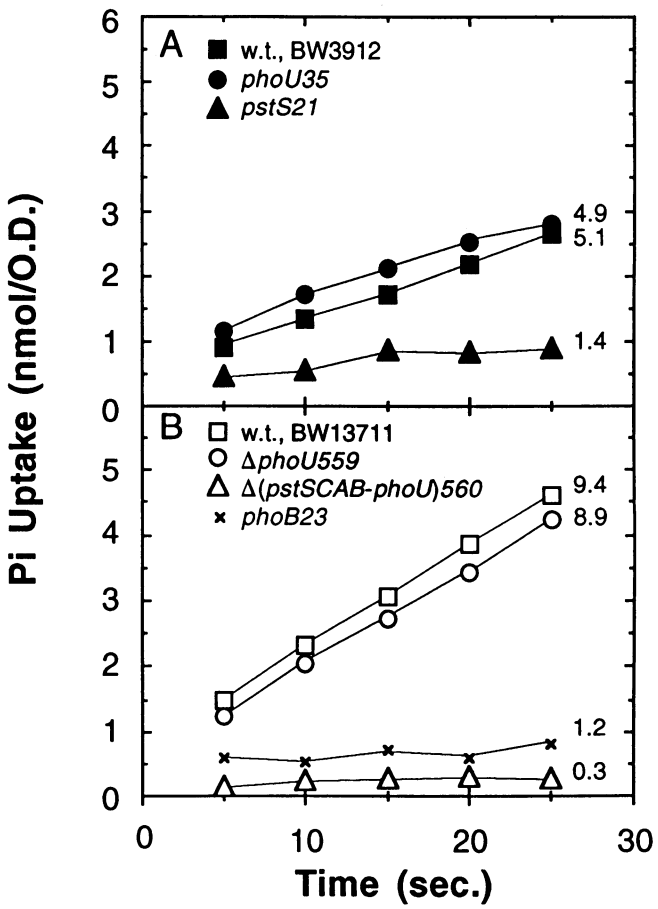


FIG. 6. $P_i$ uptake in wild-type and mutant strains. (A) BW3912 (wild type [w.t.]), BW6504 (*phoU35*), and BW6833 (*pstS21*) were grown in 0.4% glucose–MOPS containing 0.1 mM $P_i$, and uptake was measured as described in Materials and Methods. (B) BW13711 (wild type), BW14087 (*phoB23*), BW17142 (Δ*phoU559*), and BW17335 [Δ(*pstSCAB-phoU*)560] were similarly grown and assayed. The numbers adjacent to each curve are averages (nmol/O.D./min) of two or more determinations for each condition. O.D., optical density.

## DISCUSSION

Mutants carrying defined mutations of the *pstSCAB-phoU* operon were made by using M13 recombinant phage and the *rep* technique for allele replacement. This method may be generally useful, for it is simple and efficient. It involves two steps. In the first step, recombinants in which the phage has integrated into homologous chromosomal sequences by one crossover event are selected. In the second step, recombinants in which M13 has been excised by a second crossover event are selected. About half of these excision recombinants usually carried the desired mutation, because M13 phage that had similar lengths of chromosomal DNA flanking each mutation were used (Fig. 3). Also, because the antibiotic resistance gene used for selection of the M13 integrants could lie outside the regions of homology, this method allowed for exchange of unmarked mutations as well as mutations marked with an antibiotic resistance gene. Importantly, this method has been successfully used in this study for the construction of mutants

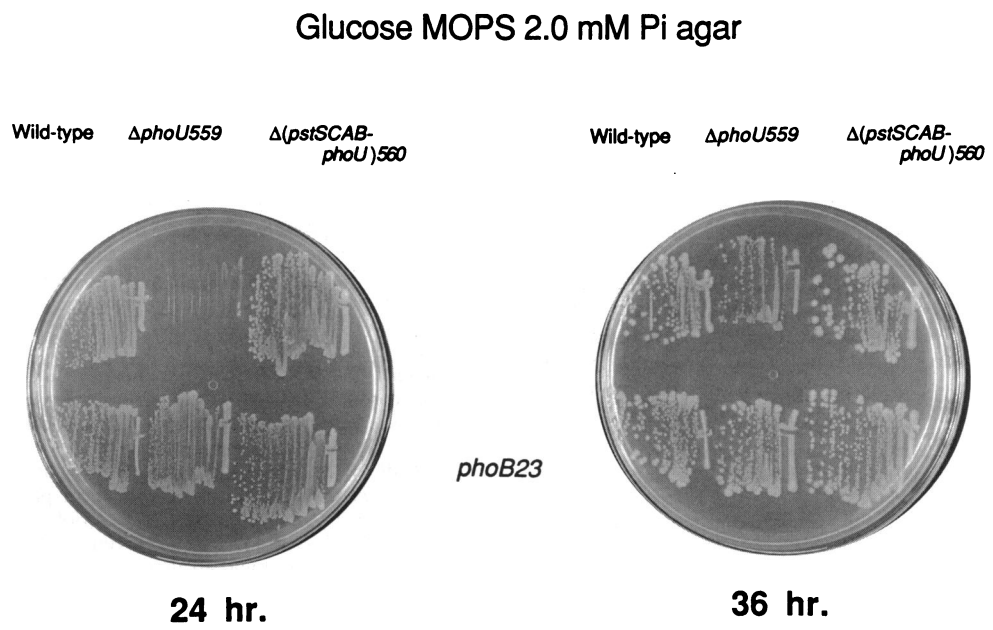


FIG. 7. Effect of a Δ*phoU* mutation on growth. Cells were streaked onto 0.2% glucose–MOPS agar containing 2 mM $P_i$ and incubated at 37°C for 24 or 36 h. Strains in the top row are *phoB*$^+$; those in the bottom row are *phoB*. Strains shown include BW13711 (wild type), BW17142 (Δ*phoU559*), BW17335 [Δ(*pstSCAB-phoU*)*560*], BW14087 (*phoB23*), BW17152 (Δ*phoU559 phoB23*), and BW17448 [Δ(*pstSCAB-phoU*)*560 phoB23*].

carrying extremely deleterious Δ*phoU* mutations. In contrast, attempts to transfer a Δ*phoU* mutation engineered in a plasmid onto the chromosome by other methods have been unsuccessful (9).

Because the PstSCAB transporter and PhoU protein are involved in negative control of the PHO regulon, deletions in the *pstSCAB-phoU* operon had the expected effect on PHO regulon control in that they abolished $P_i$ repression. Also, Δ(*pstSCAB-phoU*) and Δ(*pstB-phoU*) mutations abolished $P_i$ uptake by the PstSCAB transporter, while a Δ*phoU* mutation had no effect (Fig. 6). Therefore, the PhoU protein has no role in $P_i$ transport. Also, even though expression of the *pstSCAB-phoU* operon is repressed by $P_i$ in wild-type cells, an intact PstSCAB transporter probably exists in repressed cells. Otherwise, *pstSCAB-phoU* mutations would be unlikely to affect $P_i$ control. Therefore, an intact PstSCAB transporter could exist in a complex with the PhoU protein under conditions of repression, and this repressor complex could negatively regulate the PHO regulon as described elsewhere (33).

Unexpectedly, a Δ*phoU* mutation had an extremely deleterious effect on growth (Fig. 7). In contrast, a *phoU* mutant with the *phoU35* (missense) allele does not exhibit a severe growth defect, even though it is also fully derepressed for the PHO regulon. As a consequence, mutants containing a compensatory mutation rapidly accumulate in Δ*phoU* mutant cultures. These include ones with mutations in the *pstSCAB* genes, the *phoB* gene, and the *phoR* gene. Importantly, a deletion of the *pstB* or *pstSCAB* genes was shown to (largely) alleviate the growth defect due to a Δ*phoU* mutation. Therefore, the growth defect of a Δ*phoU* mutant is due to synthesis of a functional PstSCAB transporter in the absence of the PhoU protein. Also, defined *phoB* and *phoR* mutations alleviate the growth defect due to a Δ*phoU* mutation (this study and data not shown). A *phoB* or *phoR* mutation probably alleviates the growth defect by reducing expression of the *pstSCAB* genes.

These results lead us to propose that the PhoU protein has a new role in the PHO regulon, in addition to its role as a negative regulator. This role is required for normal growth in the presence of a functional PstSCAB transporter. Further, we propose that its new role may be that of an enzyme (or a subunit of an enzyme) involved in intracellular $P_i$ metabolism. An important role for the PhoU protein in the presence of the PstSCAB transporter is underscored by the severe growth defect of Δ*phoU* mutants that constitutively synthesize a functional PstSCAB transporter. This was shown by the severe growth defect caused by a Δ*phoU* mutation and by the absence of a severe growth defect due to a Δ(*pstSCAB-phoU*) or Δ(*pstB-phoU*) mutation. This was also shown by the relief of the severe growth defect in a Δ*phoU* mutant by a *phoB* or *phoR* mutation, which affects synthesis of the PstSCAB transporter.

Once $P_i$ is taken up, it is incorporated into ATP, the primary phosphoryl donor in metabolism (32). Accordingly, the PhoU protein may be an enzyme for ATP synthesis. It may be especially important for such an enzyme to be closely associated with the PstSCAB transporter. Such an association may be necessary for maintaining balanced growth under conditions of $P_i$ limitation. Of course, the PhoU protein itself might not have enzymatic activity. Instead, the PhoU protein could be a regulatory subunit of an enzyme for synthesis of ATP, or another organophosphate, for entry of $P_i$ into central metabolism. Also, this new role for the PhoU protein may be separate from its role as a negative regulator. This is suggested by the observation that the *phoU35* allele does not cause a severe growth defect and yet it is completely defective in $P_i$ repression. Nevertheless, further studies are necessary to determine whether the PhoU protein has two distinct roles in the PHO regulon, because the *phoU35* mutation may have a linked compensatory mutation that has no effect on $P_i$ uptake.

While this article was in preparation, a Δ*phoU* mutant having different properties was reported by Muda et al. (21). In

particular, their Δ*phoU* mutation abolished uptake, and they therefore concluded the PhoU protein had a role in $P_i$ transport. Our results are in contradiction to theirs. Since they observed no growth defect, they may have studied a Δ*phoU* mutant containing a compensatory mutation(s) in the *pstSCAB* genes. In contrast, our Δ*phoU* mutations caused a severe growth defect and had no effect on $P_i$ transport. Also, our Δ*phoU* mutants were fully complemented by a λ phage carrying the *phoU*$^+$ gene alone.

The mutants described here should be useful in new studies on the PstSCAB transporter and PhoU protein. They should be especially useful in genetic studies on how these gene products interact. New studies are directed towards understanding the presumed interactions between the PstSCAB transporter, the PhoU protein, and the PhoR protein and the proposed role for the PhoU protein in $P_i$ metabolism.

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