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Use of New Methods for Construction of Tightly Regulated Arabinose and Rhamnose Promoter Fusions in Studies of the *Escherichia coli* Phosphate Regulon

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Escherichia coli **genes regulated by environmental inorganic phosphate (P_i) levels form the phosphate (Pho) regulon. This regulation requires seven proteins, whose synthesis is under autogenous control, including response regulator PhoB, its partner, histidine sensor kinase PhoR, all four components of the P_i-specific transport (Pst) system (PstA, PstB, PstC, and PstS), and a protein of unknown function called PhoU. Here we examined the effects of uncoupling PhoB synthesis and PhoR synthesis from their normal controls by placing each under the tight control of the arabinose-regulated P_{araB} promoter or the rhamnose-regulated P_{rhaB} promoter. To do this, we made allele replacement plasmids that may be generally useful for construction of P_{araB} or P_{rhaB} fusions and for recombination of them onto the *E. coli* chromosome at the *araCBAD* or *rhaRSBAD* locus, respectively. Using strains carrying such single-copy fusions, we showed that a P_{rhaB} fusion is more tightly regulated than a P_{araB} fusion in that a P_{rhaB}-*phoR*$^+$ fusion but not a P_{araB}-*phoR*$^+$ fusion shows a null phenotype in the absence of its specific inducer. Yet in the absence of induction, both P_{araB}-*phoB*$^+$ and P_{rhaB}-*phoB*$^+$ fusions exhibit a null phenotype. These data indicate that less PhoR than PhoB is required for transcriptional activation of the Pho regulon, which is consistent with their respective modes of action. We also used these fusions to study PhoU. Previously, we had constructed strains with precise Δ*phoU* mutations. However, we unexpectedly found that such Δ*phoU* mutants have a severe growth defect (P. M. Steed and B. L. Wanner, J. Bacteriol. 175:6797–6809, 1993). They also readily give rise to compensatory mutants with lesions in *phoB*, *phoR*, or a *pst* gene, making their study particularly difficult. Here we found that, by using P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, or P_{rhaB}-*phoR*$^+$ fusions, we were able to overcome the extremely deleterious growth defect of a Pst$^+$ Δ*phoU* mutant. The growth defect is apparently a consequence of high-level Pst synthesis resulting from autogenous control of PhoB and PhoR synthesis in the absence of PhoU.**

The control of the *Escherichia coli* phosphate (Pho) regulon by environmental inorganic phosphate (P_i) levels is a paradigm of a bacterial signal transduction pathway in which occupancy of a cell surface receptor (the P_i-specific binding protein PstS) regulates gene expression in the cytoplasm (reference 29 and references therein). This signal transduction pathway requires seven proteins, all of which probably interact in a membrane-associated signaling complex. The P_i signaling proteins include (i) two members of the large family of two-component regulatory systems, namely, response regulator PhoB (a transcriptional activator) and its partner, histidine sensor kinase PhoR (itself an integral-membrane protein); (ii) four components of the ATP-binding cassette family P_i-specific transport (Pst) machinery (PstA, PstB, PstC, and PstS); and (iii) a negative regulator of unknown function called PhoU.

We proposed elsewhere that the P_i signaling response involves three processes: activation, deactivation, and inhibition (30). Accordingly, activation occurs under conditions of P_i limitation and requires both PhoB and PhoR; activation involves autophosphorylation of PhoR by ATP, phosphotransfer to PhoB, and transcriptional activation of Pho regulon promoters by phospho-PhoB (P-PhoB). Deactivation is a distinct intermediate step-down process that occurs upon a growth shift from P_i-limiting to P_i excess conditions. It is required to reestablish inhibition and leads to the dephosphorylation of P-PhoB in a process requiring PhoR and an excess of either PhoU, a Pst component(s), or both. Inhibition prevents phosphorylation of PhoB when P_i is in excess; it requires all seven P_i signaling proteins (PhoB, PhoR, PhoU, PstA, PstB, PstC, and PstS) in an "inhibition complex" that, by insulating PhoB, interferes with its phosphorylation.

In order to study how PhoU and the Pst system interact with PhoB and PhoR, we had previously constructed mutants with defined deletions of the *pstSCAB-phoU* operon. This led to our discovery that a Δ*phoU* (unlike a *phoU* missense) mutation causes a severe growth defect, resulting from an apparent P_i sensitivity phenotype (26). Growth inhibition by P_i has also been seen in mutants of the Pst transporter in which the P_i transport channel is permanently "switched on" (open), although in that case the growth defect is much less severe (34). The deleterious growth phenotype resulting from a Δ*phoU* mutation also leads to the accumulation of compensatory mutants with lesions in *phoB*, *phoR*, or a *pst* gene under normal growth conditions (26). Indeed, the poor growth of a Δ*phoU* mutant was apparently responsible for another laboratory concluding incorrectly that a Δ*phoU* mutation abolishes P_i uptake (19), as their "Δ*phoU* mutant" carries also a linked *pst* mutation (29). On the contrary, we proved that a Δ*phoU* mutation has no effect on P_i uptake (26).

We have now found ways to overcome the difficulties of studying *phoU* and (presumably) open-channel *pst* mutations as well. The growth defect due to a Δ*phoU* mutation is appar-

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ent only when the Pst system is synthesized at a high level, which in turn requires increased amounts of PhoB and PhoR, whose synthesis is autogenously controlled (10). We uncoupled *phoB* expression and *phoR* expression from their normal controls by placing them behind the foreign, arabinose-regulated P_{araB} or rhamnose-regulated P_{rhaB} promoter. Upon induction of the corresponding Δ*phoB* or Δ*phoR* mutant with arabinose or rhamnose, such strains show nearly normal P_i control of the Pho regulon. However, the fold induction is lowered. Importantly, a Δ*phoU* mutation has no deleterious effect on growth of these strains, even in the presence of the respective inducer. Our methods for constructing and characterizing P_{araB} and P_{rhaB} fusions may also be generally useful.

MATERIALS AND METHODS

Media and culture conditions. Luria-Bertani broth, tryptone-yeast extract, and M63 were routinely used as complex and minimal media. These were prepared as described elsewhere (28). To maintain plasmids, antibiotics (Sigma, St. Louis, Mo.) were added as follows: ampicillin at 100 μg/ml, gentamicin at 15 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 12.5 μg/ml. Recombinants with a single-copy plasmid were selected with gentamicin at 4 μg/ml or kanamycin at 10 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Bachem, Torrance, Calif.) was used at 40 μg/ml to detect β-galactosidase. Tetracycline-sensitive (Tet^s) cells were selected on tetracycline-sensitive-selective (TSS) agar as described elsewhere (17). MacConkey agar (Difco, Detroit, Mich.) containing 1% L-arabinose, lactose, or L-rhamnose (Sigma) was used to test for the use of these carbon sources. Ara^- and Rha^- strains were verified by their inability to grow on M63 media containing 0.2% L-arabinose or L-rhamnose, respectively. Ara^- and Rha^- recombinants are distinguishable from arabinose- or rhamnose-sensitive ones by the inability of the latter to grow on MacConkey or glycerol agar in the presence of arabinose or rhamnose, respectively. In contrast, Ara^- and Rha^- recombinants are insensitive to these carbohydrates.

MOPS (morpholinepropanesulfonic acid) media containing different carbon sources were used to study gene regulation in P_{araB}, P_{rhaB}, and P_{rhaS} fusion strains. Cells were grown on MOPS agar containing the same carbon source at 0.2% for D-glucose, D-fructose, and D-mannitol or 0.4% for glycerol, but without an inducer. Fresh isolated colonies (after less than 20 h of growth) were used to inoculate 0.06% glucose–, fructose–, or mannitol–MOPS medium or 0.1% glycerol–MOPS medium without or with an inducer. Cultures were incubated at 37°C for 16 to 24 h prior to the assay. Such carbon-limited cultures yield highly reproducible values that are qualitatively similar to ones obtained for logarithmic-growth phase cultures (27). L-Arabinose, L-rhamnose, and isopropyl-β-D-galactopyranoside (IPTG; Sigma) were used at 1.3, 1.1, and 0.2 mM, respectively, for induction.

Bacteria. All strains assayed are described in Table 1. Others included BT333 (*endA*::*tetAR*; from W. Wackernagel [4]), BW5045 (*srlC300*::Tn*10*; [18]), BW8078 (λ*recA*$^+$ *recA1*; [28]), BW21391 (*leu-63*::Tn*10*; [12]), BW22773 (*lacI*^{q} $rrnB_{T14}$ Δ*lacZ*$_{WJ16}$ *proC*::Tn*5-132*; [14]), CA10 (*galU95*; from M. Berlyn), JW383 (*metF159 zii-510*::Tn*10 thi-1*; from M. Berlyn), W3110*trpB114* (*trpB114*::Tn*10*; from C. Yanofsky), and ZK1001 (*cysC95*::Tn*10 rpoS*::*kan*; from R. Kolter). Only relevant markers are given in parentheses.

Plasmids and phage. pAH85, pSK49, and pSK58 were constructed in an earlier study; each has the same backbone as pSK*50*Δ*uidA2* (13). pAH85 and pSK58 are similar, except that the former has a silent *Spe*I site at codon 113 of *phoB*. Each expresses *phoB* from its native promoter. pSK49 expresses *phoB* from P_{tac}. pAH136 and pAH150 are derivatives of pAH85 and pSK49 carrying P_{rhaB} and P_{araB} in place of P_{phoB} and the *lacI*^{q}-P_{tac} region, respectively; pAH151 and pAH152 are similar plasmids carrying P_{rhaB}-*phoR*$^+$ and P_{rhaS}-*phoR*$^+$ except that they have the attachment site of HK022 and encode gentamicin resistance (14). pBAD32 and pBAD33 (11) were from L.-M. Guzman. They are similar except that pBAD32 has an undefined deletion of ca. 0.6 kbp between the polylinker and the *cat* gene of pBAD33 (data not shown). pBC35 is a derivative of pBGS19+ (25) carrying the *phoBR* operon within a 4.7-kbp *Pst*I-to-*Eco*RI fragment (32) (Fig. 1). pSK47 (16) has the same *phoBR* fragment cloned into the backbone of pSK50Δ*uidA2* (13). pSLF13 and pSLF22 were constructed in an earlier study (8). pSLF13 is a derivative of pSPORT1 (Gibco-BRL, Bethesda, Md.) that encodes a segment of VanS within a 0.4-kbp *Eco*RI-to-*Bam*HI insert that has a start codon overlapping an *Nde*I site immediately downstream of a synthetic ribosome-binding site. pSLF22 is a derivative of pBAD32 containing a similar insert. pWJ17 and pWJ18 are derivatives of the *lacZ* transcriptional fusion vector pWJ13 containing a kanamycin resistance cassette as a *Pst*I fragment, whose loss facilitates recognizing promoter-*lacZ* fusion plasmids as kanamycin-sensitive (Kan^s) derivatives (15). Fusions made with these plasmids were recombined onto the chromosome by allele replacement as described below.

All plasmids constructed in this study are described in Table 2. Many have the γ replication origin of R6K, denoted $oriR_{R6K\gamma}$, which requires the Π protein (encoded by *pir*) for replication as a plasmid. Many also contain *tetAR*, which can be deleterious when present at high copy number. Therefore, these plasmids were routinely maintained in BW23473 or BW24249 or in similar *pir*$^+$ hosts (17). λRZ*5lacP-phoU*$^+$ (PS15) has been described previously (26). Generalized transduction was carried out with P1*kc* from a laboratory stock.

Molecular biology methods. PCR amplifications of *ara*, *rha*, and *phoR* sequences were carried out with Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and oligonucleotide primers (Table 3; IDT Inc., Coralville, Iowa). Other enzymes were from New England Biolabs or Promega (Madison, Wis.). QIAGEN (Hilden, Germany) products were used for isolation of plasmid DNA, extraction of DNA fragments from agarose gels, and purification of PCR fragments. The *phoR*, P_{araB}, and P_{rhaSB} fragments were sequenced on both strands at the Dana Farber Cancer Institute Molecular Biology Core Facility (Harvard Medical School, Boston, Mass.). The '*araD'* and '*rhaD* fragments (the prime indicates that a gene portion is missing on the side of the prime) were verified only by restriction enzyme analysis.

Molecular genetics. Many new strains were constructed by generalized P1 transduction as described elsewhere (28). BW23321 (Δ*lac-169 hsdR514 uidA* (Δ*Mlu*I)::*pir*$^+$ $endA_{BT333}$) was made by transduction of BW21116 (17) to tetracycline resistance (Tet^r) with P1 grown on BT333 followed by transformation of resultant transductant BW23298 with FLP expression plasmid pCP20 and excision of the *tetAR* genes as described elsewhere (4). The notation $endA_{BT333}$ signifies the resultant allele. In order to construct strains carrying a minimum number of antibiotic resistance markers, new mutations were usually introduced in two steps. In the first step, a linked auxotrophic marker was introduced by selecting Tet^r transductants. These were then made prototrophic with P1 grown on an appropriate donor; the resultant transductants were scored for loss of antibiotic resistance and other relevant phenotypes. Several new mutations and fusions were constructed on plasmids as described above and then recombined onto the chromosome by allele replacement as described elsewhere (17). The resultant chromosomal allele is often given a designation corresponding to the plasmid used in its construction. As necessary, transductants carrying a Δ*phoU* mutation were verified by complementation with λRZ*5lacP-phoU*$^+$ upon introduction of *phoB*$^+$ or *phoR*$^+$ or by backcross of the Δ*phoU* mutation into an appropriate recipient.

The Δ*phoB578* mutation (Fig. 1) was recombined onto the chromosome of BW21016 [DE3(*lac*)$_{X74}$] by using pLD83 to make BW22901. The P_{rhaB}-*lacZ*$_{LD68}$ and P_{rhaS}-*lacZ*$_{LD69}$ fusions were recombined onto the chromosome of BW21578 ($rrnB_{T14}$ Δ*lacZ*$_{WJ16}$) by using pLD68 and pLD69 to make BW22716 and BW22721, respectively. The P_{araB}-*lacZ*$_{AH31}$ fusion was recombined onto the chromosome of BW21480 (*lacI*^{q} $rrnB_{T14}$ Δ*lacZ*$_{WJ16}$) by using pAH31 to make BW22746. The desired *lacZ* fusion recombinants were recognized as ones showing a rhamnose- or arabinose-dependent Lac^+ phenotype on X-Gal agar. These fusions have the following on the chromosome at the *lac* locus in a counterclockwise orientation: *lacI*, four tandem copies of the *rrnB* transcriptional terminator (denoted $rrnB_{T14}$), and the foreign promoter preceding the *lacZYA* operon. The construction of these and similar strains with *lacI*^{q} $rrnB_{T14}$ P_{phnC}-*lacZ*$_{WJ19}$, *lacI*^{q} $rrnB_{T14}$ Δ*lacZ*$_{WJ16}$, $rrnB_{T14}$ Δ*lacZ*$_{WJ16}$, and other promoter-*lacZ* fusions is unpublished (15). Site-specific recombination of $oriR_{R6K\gamma}$ *attP* plasmids onto the chromosome has been described previously (13). All integrants were verified by PCR as described elsewhere (12).

The P_{araB} and P_{rhaB} fusions were recombined onto the chromosome by using derivatives of new allele replacement plasmids pAH33, pAH54, and pLD78 (Fig. 2). This was done with an Ara^+ or Rha^+ parental strain, so that the resulting segregants with an allele replacement were recognizable as Ara^- or Rha^- recombinants, respectively. Because these plasmids contain a segment of *araD* or *rhaD*, most integrants formed by the initial recombination event are $AraD^-$ or $RhaD^-$. Such recombinants are arabinose or rhamnose sensitive, respectively. All selections are therefore carried out in the absence of arabinose or rhamnose.

The Δ$araBAD_{AH33}$ mutation was recombined onto the chromosome of BW13711 [DE3(*lac*)$_{X74}$] by using pAH33. Integrants carrying pAH33 were selected as Tet^r transformants; they were purified once nonselectively, after which Tet^s segregants were selected on TSS agar. One showing the expected Ara^- phenotype was verified and named BW22826. Similarly, the P_{araB}-*phoR*[M1-D431]$_{AH35}$ fusion was recombined onto the chromosome of BW21555 (Δ*phoR574*) by using pAH35 to make BW22835. Recombination of the P_{araB}-*phoR*[M1-D431]$_{AH35}$ fusion onto the chromosome results also in removal of the same sequences eliminated by the Δ$araBAD_{AH33}$ mutation. Hence, the resultant allele is denoted Δ$araBAD_{AH33}$::P_{araB}-*phoR*[M1-D431]$_{AH35}$. The Δ$rhaBAD_{LD78}$ mutation and Δ$rhaBAD_{LD78}$::P_{rhaB}-*phoB*$_{LD79}$ fusion were recombined onto the chromosome of BW22860 [Δ(*phoBR brnQ*)*525* Δ*cya-161*] by using pLD78 and pLD79 to make BW22875 and BW22876, respectively. The desired recombinants were recognized as Rha^- ones.

Enzyme assays. β-Galactosidase and bacterial alkaline phosphatase (BAP) assays were carried out as described elsewhere (28).

RESULTS

Construction of allele replacement vectors for tightly regulated protein synthesis from arabinose- and rhamnose-regulated promoters. Since expression of the *phoBR* operon is subject to positive autogenous control (10), we considered that

TABLE 1. Bacterial strains

Strain[a]	Genotype	Pedigree[b]	Source or reference(s)
BW17142	DE3(lac)$_{X74}$ Δ*phoU559*::*kan rpoS*(Am)	BD792 via BW13711	26
BW17335	DE3(lac)$_{X74}$ Δ(*pstSCAB-phoU*)*560*::*kan rpoS*(Am)	BD792 via BW13711	26
BW18897	DE3(lac)$_{X74}$ Δ*phoU559 rpoS*(Am) *phn*(*Eco*B)	BD792 via BW18834	Ilv$^+$ with P1 on BW18833 (26)
BW21578	*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$	BD792 via BW21342	15
BW22831	*lacI*^{q} *rrnB*$_{T14}$ *P*$_{araB}$-*lacZ*$_{AH31}$ Δ*araBAD*$_{AH33}$ *rpoS*(Am)	BD792 via BW22766	Leu$^+$ Ara$^-$ with P1 on BW22826[c] (12)
BW22861	*lacI*^{q} *rrnB*$_{T14}$ *P*$_{phnC}$-*lacZ*$_{WJ19}$ Δ*phoR574* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(*Eco*B) Δ*araBAD*$_{AH33}$::*P*$_{araB}$-*phoR*[M1-D431]$_{AH35}$ *rpoS*(Am)	BD792 via BW22829	Leu$^+$ Ara$^-$ with P1 on BW22835
BW22886	*rrnB*$_{T14}$ *P*$_{rhaB}$-*lacZ*$_{LD68}$ Δ*rhaBAD*$_{LD78}$	BD792 via BW22779	Met$^+$ Rha$^-$ with P1 on BW22875[d]
BW22887	*rrnB*$_{T14}$ *P*$_{rhaB}$-*lacZ*$_{LD68}$ Δ*rhaBAD*$_{LD78}$ *rpoS*(Am)	BD792 via BW22780	Met$^+$ Rha$^-$ with P1 on BW22875
BW22888	*rrnB*$_{T14}$ *P*$_{rhaS}$-*lacZ*$_{LD69}$ Δ*rhaBAD*$_{LD78}$	BD792 via BW22781	Met$^+$ Rha$^-$ with P1 on BW22875
BW23473	Δ*lac-169 rpoS*(Am) *robA1 creC510 hsdR514 uidA*(Δ*Mlu*I)::*pir*$^+$ *endA*$_{BT333}$ *recA1*	BD792 via BW23438	12, 13
BW24249	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoBR580* Δ*creABCD154 hsdR514* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(*Eco*B) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ *uidA*(Δ*Mlu*I)::*pir*$^+$ *rposS*(Am) *endA*$_{BT333}$ *galU95 recA1*	BD792 via BW24217	Srl$^+$ *recA* with P1 on BW8078 (28)
BW24486	*lacI*^{q} *rrnB*$_{T14}$ *P*$_{phnC}$-*lacZ*$_{WJ19}$ Δ*phoBR580* Δ*creBCD153* Δ(*pta ackA hisQ hisP*)$_{TA3516}$ *phn*(*Eco*B) Δ*araBAD*$_{AH33}$::*P*$_{araB}$-*phoR*[M1-D431]$_{AH35}$ *rpoS*(Am) Δ(*pstSCAB-phoU*)*560*::Ω	BD792 via BW23424	Spc^r and Str^r with P1 on BW17636 (26)
BW24508	Like BW24486 except *att*$_λ$::pSK58(*P*$_{phoB}$-*phoB*$^+$)	BD792 via BW24486	pSK58 integrant
BW24962	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoB578* Δ*creABDC154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$	BD792 via BW24651	Pro$^+$ with P1 on BW23562 (13)
BW24739	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*creABDC154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ(*pstSCAB-phoR*)*560*::Ω	BD792 via BW24713	Ilv$^+$ with P1 on BW17646 (26)
BW24740	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoB574* Δ*creABDC154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ(*pstSCAB-phoU*)*560*::Ω	BD792 via BW24715	Ilv$^+$ with P1 on BW17646 (26)
BW24741	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoB578* Δ*creABDC154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ(*pstSCAB-phoU*)*560*::Ω	BD792 via BW24717	Ilv$^+$ with P1 on BW17646 (26)
BW24768	Like BW24740 except *att*$_{HK022}$::pAH151(*P*$_{rhaB}$-*phoR*$^+$)	BD792 via BW24740	pAH151 integrant
BW24770	Like BW24741 except *att*$_λ$::pAH85(*P*$_{phoB}$-*phoB*$^+$)	BD792 via BW24741	pAH85 integrant
BW24773	Like BW24741 except *att*$_λ$::pAH136(*P*$_{rhaB}$-*phoB*$^+$)	BD792 via BW24741	pAH136 integrant
BW24774	Like BW24741 except *att*$_λ$::pAH150(*P*$_{araB}$-*phoB*$^+$)	BD792 via BW24741	pAH150 integrant
BW24775	Like BW24741 except *att*$_λ$::pSK49(*P*$_{tac}$-*phoB*$^+$)	BD792 via BW24741	pSK49 integrant
BW24777	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoB578* Δ*creABCD154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ(*pstSCAB-phoU*)*560*::Ω Δ*rhaBAD*$_{LD78}$::*P*$_{rhaB}$-*phoB*$_{LD79}$	BD792 via BW24754	Met$^+$ Rha$^-$ with P1 on BW22876
BW24803	Like BW24692 except *att*$_λ$::pAH150(*P*$_{araB}$-*phoB*$^+$)	BD792 via BW24692	pAH150 integrant
BW24858	Like BW24740 except *att*$_{HK022}$::pAH152(*P*$_{rhaS}$-*phoR*$^+$)	BD792 via BW24740	pAH152 integrant
BW24936	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoR574* Δ*creABCD154 rpoS*(Am) Δ*araBAD*$_{AH33}$ ΔrhaBAD$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ*phoU559*	BD792 via BW24714	Ilv$^+$ Spc^s and Str^s with P1 on BW24627
BW24937	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoB578* Δ*creABCD154 rpoS*(Am) Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ(*ackA pta*)*160*$_{AH25}$ Δ*phoU559*	BD792 via BW24716	Ilv$^+$ Spc^s and Str^s with P1 on BW24627
BW24948	Like BW24936 except *att*$_{HK022}$::pAH151(*P*$_{rhaB}$-*phoR*$^+$)	BD792 via BW24936	pAH151 integrant
BW24949	Like BW24937 except *att*$_λ$::pAH136(*P*$_{rhaB}$-*phoB*$^+$)	BD792 via BW24937	pAH136 integrant
BW24950	Like BW24937 except *att*$_λ$::pAH150(*P*$_{araB}$-*phoB*$^+$)	BD792 via BW24937	pAH150 integrant

[a] All are derivatives of *E. coli* K-12, except that the *phn*(*Eco*B) locus is from *E. coli* B (31).

[b] Pedigree gives the parental strain from another laboratory and its most immediate ancestor in this laboratory. Many of our strains are descendents of *E. coli* K-12 strain BD792 (CGSC6159), which, like MG1655 (CGSC6300), is an unmutagenized and direct descendent of *E. coli* W1485 (CGSC5024) (1, 2).

[c] The *ara* and *leu-63*::Tn*10* markers are about 55% linked in P1*kc* crosses (data not shown).

[d] The relevant gene order is *zii-510*::Tn*10 metF159* Δ*rhaBAD*$_{LD78}$ in which Tn*10* and *metF* are ca. 70% linked and *metF* and *rha* are ca. 10% linked (data not shown).

it would be advantageous to uncouple PhoB synthesis and PhoR synthesis from their normal controls for new studies on the Pho regulon. We therefore constructed a derivative of P_{araB} plasmid pBAD32 (11) carrying a P_{araB}-*phoR*$^+$ fusion (pAH21; Table 2). However, in preliminary studies, we found that substantial amounts of PhoR were apparently made in the absence of inducer. A Δ*phoR* mutant carrying pAH21 synthesized BAP upon P_i limitation in the absence of arabinose even under conditions of catabolite repression due to glucose (data not shown). No doubt the "leakiness" of P_{araB} under these conditions reflects the small amount of PhoR required for activation (phosphorylation) of PhoB. To further reduce PhoR synthesis, we assembled an allele replacement, "suicide" vector system to recombine the P_{araB}-*phoR*$^+$ fusion onto the chromosome in single copy at the *ara* locus. We also constructed an analogous allele replacement vector carrying the rhamnose-regulated P_{rhaB} promoter (6) for construction and recombina-

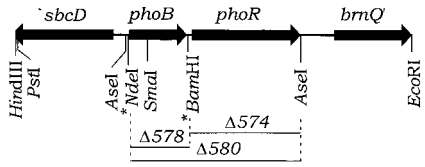

FIG. 1. Structure of the *phoBR* chromosomal region. The 4.7-kbp *Pst*I-to-*Eco*RI fragment in pBC35 and pSK47 is shown. Asterisks mark the *Nde*I and *Bam*HI sites that were previously introduced by site-directed mutagenesis upstream and downstream, respectively, of the *phoB* coding region. The Δ*phoB578* mutation was made in pLD82 (Table 2) and recombined onto the chromosome by allele replacement as described in the text. The construction of Δ*phoR574* and Δ*phoBR580* is described elsewhere (12). Arrows show gene orientations.

tion of a P_{rhaB}-*phoB*$^+$ fusion onto the chromosome in single copy at the *rha* locus.

Our vectors for constructing chromosomal P_{araB} and P_{rhaB} fusions are illustrated in Fig. 2. pAH33 and pAH54 are useful for making a P_{araB} fusion; pLD78 is useful for making a P_{rhaB} fusion. These plasmids contain sequences of the *araCBAD* or *rhaRSBAD* locus flanking cloning sites for construction of the respective promoter fusion. pAH33 and pAH54 have a 0.5-kbp fragment containing P_{araB} and a 0.6-kbp fragment of *araD* upstream and downstream of the cloning region, respectively. They differ in that pAH33 has two *Nde*I sites (one in *tetR* and the other in a segment of *lacZ*) and pAH54 has only one *Nde*I site (in *tetR*; Table 2). Derivatives of the latter have facilitated the use of *Nde*I in particular plasmid constructions (data not shown). pLD78 has a 0.3-kbp fragment containing P_{rhaB} and a 0.6-kbp fragment of *rhaD* upstream and downstream of its cloning region, respectively. The flanking upstream and downstream sequences provide homologous regions for recombining the fusions onto the chromosome by allele replacement.

pAH33, pAH54, and pLD78 are derivatives of pWJ18 (or pWJ17; Fig. 2), which is in turn a derivative of *pir*-dependent, counterselectable (*tetAR*) allele replacement vector pLD53 (17). Upon introduction of these plasmids into a normal (non-*pir*) Ara$^+$ host (by transformation, electroporation, or conjugation), they cannot replicate and therefore integrate into the chromosome via homologous recombination. Figure 3 shows the integration of P_{araB}-*phoR*$^+$ plasmid pAH35 (Table 2) at the *araCBAD* locus. Integrants are selectable as Tet^r colonies. A subsequent recombination event occurring in the absence of selection leads to the loss of plasmid sequences; this event results in restoration of the parental (wild-type) chromosomal structure or an allele replacement. Recombinants that have lost the plasmid backbone are selectable as Tet^s ones. Those carrying the desired chromosomal P_{araB} or P_{rhaB} fusion have the fused gene in single copy at the *araCBAD* or *rhaRSCAB* locus in place of *araBAD* or *rhaBAD* sequences (Fig. 4); they are therefore recognizable as Ara$^-$ or Rha$^-$ ones, respectively. Ones that are also antibiotic sensitive are then verified by genetic linkage or PCR tests. Because the appropriate segregants are Ara$^-$ or Rha$^-$, the recombinants provide the additional advantage of not being able to catabolize the respective inducer.

Measurements of arabinose and rhamnose regulation of single-copy promoter-*lacZ* fusions. To judge the regulatory capabilities of the arabinose- and rhamnose-regulated promoters in this system, we constructed P_{araB}-, P_{rhaB}-, and P_{rhaS}-*lacZ* fusions and recombined them onto the chromosome in single copy at the *lac* locus (Materials and Methods). We then exam-

TABLE 2. Plasmid constructions

Plasmid	Description[a]	Construction[b]
pAH16	P_{lac}-*phoR*$^+$ *bla ori*R_{MB1} *lacI*$^+$	Replaced 0.4-kbp *Eco*RI-to-*Bam*HI *vanS* fragment of pSLF13 with similar 1.3-kbp *phoR*$^+$ fragment generated by using P109 and P110 with pBC35 as the template
pAH21[c]	P_{araB}-*phoR*$^+$ *cat ori*R_{15A} *araC*$^+$	Replaced 0.4-kbp *Nde*I-to-*Sac*I *vanS* fragment of pSLF22 with similar 1.3-kbp *phoR*$^+$ fragment from pAH16
pAH31	P_{araB}-*lacZ tetAR bla ori*$R_{R6K\gamma}$	Replaced 0.5-kbp *Xho*I-to-*Pst*I *kan* fragment of pWJ18 with 0.5-kbp P_{araB} PCR fragment generated by using P125 and P126 with pBAD33 as the template
pAH33[d]	P_{araB}-*'araD' tetAR bla ori*$R_{R6K\gamma}$	Replaced 3.0-kbp *Sal*I-to-*Bsi*WI *lacZ* fragment of pAH31 with 0.6-kbp *'araD'* PCR fragment generated by using P127 and P128 with BW13711 chromosomal DNA as the template
pAH35	P_{araB}-*phoR*$^+$-*'araD' tetAR bla ori*$R_{R6K\gamma}$	Subcloned 1.4-kbp *Bam*HI *phoR*$^+$ fragment from pAH21 into pAH33
pAH54	Like pAH33 except lacking *Nde*I_2	Digested pAH33 with *Bsi*WI and partially with *Nde*I followed by filling-in with T4 DNA polymerase and religation
pLD68	P_{rhaB}-*lacZ tetAR bla ori*$R_{R6K\gamma}$	Replaced 1.3-kbp *Xho*I-to-*Sal*I *kan* fragment of pWJ18 with similar 0.3-kbp P_{rhaSB} fragment generated by using P119 and P120 with pLD1 (3) as the template
pLD69	P_{rhaS}-*lacZ tetAR bla ori*$R_{R6K\gamma}$	Same as pLD68, except fragment is in opposite orientation
pLD70	*'rhaD tetAR bla ori*$R_{R6K\gamma}$	Replaced 3.0-kbp *Bam*HI-to-*Bsi*WI *lacZ* fragment of pWJ17 with 0.6-kbp *'rhaD* fragment generated by using P121 and P122 with BW13711 chromosomal DNA as the template
pLD71	P_{rhaB}-*'rhaD tetAR bla ori*$R_{R6K\gamma}$	Subcloned 0.7-kbp *Xho*I-to-*Bam*HI P_{rhaB} fragment from pLD68 into pLD70
pLD77	Like pWJ17 except lacking *Nde*I_2	Digested pWJ17 partially with *Nde*I followed by filling-in with T4 DNA polymerase and religation
pLD78	Like PLD71 except lacking *Nde*I_2	Replaced 4.3-kbp *Xho*I-to-*Bsi*WI *lacZ* fragment of pLD77 with similar 0.8-kbp P_{rhaB}-*'rhaD* fragment from pLD71
pLD79	P_{rhaB}-*phoB*$^+$-*'rhaD tetAR bla ori*$R_{R6K\gamma}$	Subcloned 0.7-kbp *Nde*I-to-*Bam*HI *phoB*$^+$ fragment from pSK2 (9) into pLD78 digested with *Bam*HI and partially with *Nde*I
pLD82	Δ*phoB578 kan ori*$R_{R6K\gamma}$	Digested pSK47 with *Nde*I and *Bam*HI followed by filling-in with T4 DNA polymerase and religation
pLD83	Δ*phoB578 tetAR bla ori*$R_{R6K\gamma}$	Subcloned 3.4-kbp *Nco*I fragment from pLD82 that was made blunt with T4 DNA polymerase into *Sma*I-cut pLD55 (17)

[a] The replication origins of pMB1 and p15A are denoted *ori*R_{MB1} and *ori*R_{15A}, respectively.
[b] Additional information is given in the text. Oligonucleotide primers are shown in Table 3.
[c] pAH21 has a unique *Nde*I site at the *phoR* start codon and unique downstream *Sac*I, *Sph*I, *Pst*I, and *Hin*dIII sites.
[d] Restriction analysis showed that the *Sph*I and *Bgl*II sites in P127 were absent from the cloned fragment.

TABLE 3. Oligonucleotide primers

Name	Description	Sequence[a]
P109	*phoR* N terminus	5′-G**GAATTC**GT**CATATG**CTGGAACGGC-3′
P110	*phoR* C terminus	5′-CG**GGATCC**GCT**GAGCTC**AGTCGCTGTTTTTGGC-3′
P119	P_{rhaSB} upstream	5′-CCG**CTCGAG**G**CCATGG**TGGCCTCCTGATGTCG-3′
P120	P_{rhaSB} downstream	5′-CCG**GTCGACATATG**GTGATCCTGCTGAATTTC-3′
P121	*rhaD′* upstream	5′-CG**GGATCCGCATGC**G**TCGAC**AGCGACGG-3′
P122	*rhaD′* downstream	5′-CGTA**CGTACG**CAGCGCCAGCGCAC-3′
P125	P_{araB} downstream	5′-GCGCG**TGCAG**GTATGGAGAAACAGTAGAG-3′
P126	P_{araB} upstream	5′-GGTC**CTCGAG**CGAATGGTGAGATTG-3′
P127	*′araD′* upstream	5′-GACAC**GTCGACGCATGCAGATCT**CAAACGCCAG-3′
P128	*′araD′* downstream	5′-CGTA**CGTACG**ACCTCTTCCAGCAC-3′

[a] Underlined bases are complementary to the template(s). Those in boldface indicate restriction enzyme sites.

ined *lacZ* expression by measuring β-galactosidase levels when strains were grown in the presence and absence of the respective inducer on different carbon sources. Different carbon sources were used because these promoters are subject to catabolite repression. The results are shown in Table 4. In brief, arabinose or rhamnose led to induction of >100-fold to several thousandfold, depending upon the promoter, inducer, and carbon source. As expected, the lowest expression levels were obtained during growth on glucose, for which catabolite repression is the strongest; intermediate expression levels were obtained during growth on fructose, for which catabolite repression is less severe; the highest expression levels were obtained during growth on glycerol, for which catabolite repression is the weakest (7). Differences in both the basal (uninduced) and induced levels exist. In the absence of inducer, the basal level was about 10-fold higher for P_{araB} than for P_{rhaB} or P_{rhaS}. The induced levels were similar for P_{araB} and P_{rhaB} (compare BW22831 with BW22887), while the induced levels for P_{rhaS} were ca. 25% of those for P_{rhaB} (compare BW22886 with BW22888).

In the course of this study, we found that several of our strains carry an *rpoS*(Am) mutation (Table 1), which is also present in many lines of *E. coli* K-12, including progenitor K-12 strains EMG2 and W1485 (1, 22). As shown in Table 4, the induced level of P_{rhaB} expression is significantly lower in an $rpoS^+$ strain. Apparently, this *rpoS* effect is related to catabolite repression, as the magnitude varies with the carbon source. The reason for this is unknown. The induction levels in this study are therefore directly comparable only among strains having the same *rpoS* allele.

Strategy for using P_{araB} and P_{rhaB} fusions to study regulation of the Pho regulon. We constructed a number of Δ*phoB* and Δ*phoR* mutants in which PhoB or PhoR is synthesized under the control of a foreign inducible promoter(s). We did

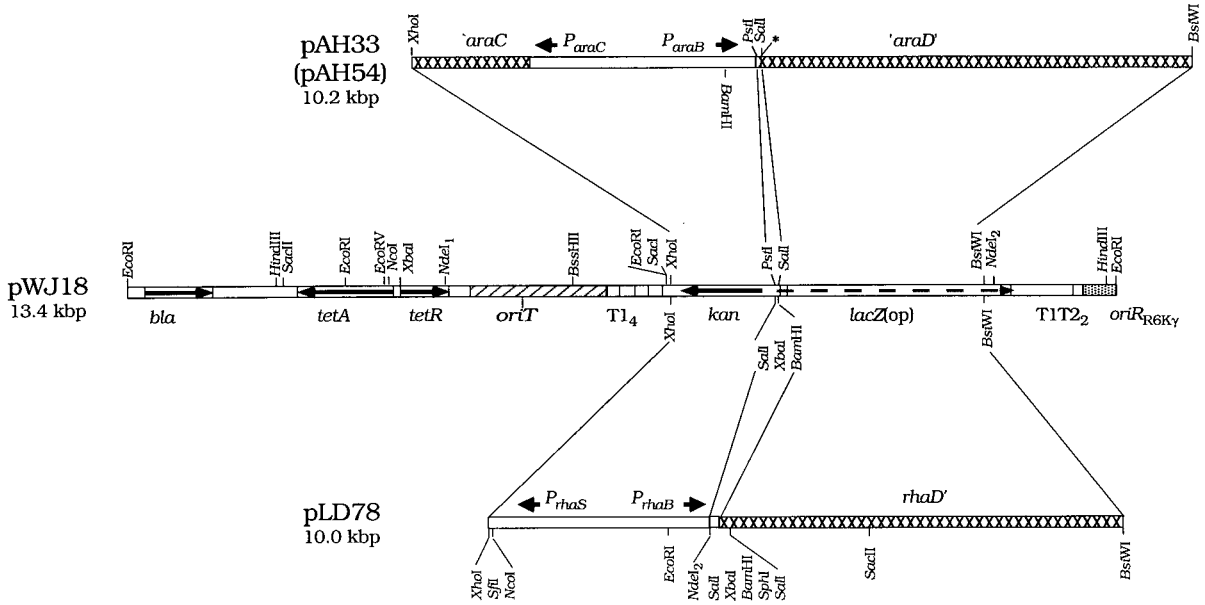

FIG. 2. Structures of the P_{araB} and P_{rhaB} allele replacement plasmids. Arrows show gene and promoter orientations. Open boxes in pAH33, pAH54, and pLD78 are promoter regions; hatched boxes are coding regions. All sites are shown for the enzymes indicated. *Nde*I sites are subscripted to correspond to those indicated in Table 2. pAH33 and pAH54 contain unique *Pst*I and *Sal*I sites downstream of P_{araB} for construction of a P_{araB} fusion. Genes cloned into these sites require also a ribosome binding site. pLD78 has *Sal*I, *Xba*I, *Bam*HI, and *Sph*I sites downstream of P_{rhaB} for construction of a P_{rhaB} fusion. pLD78 has the native *rhaB* ribosome-binding site upstream of the $NdeI_2$ site, which corresponds to the normal *met* start codon of *rhaB*. Therefore, genes cloned into this site do not require a ribosome-binding site, although partial digestions are required to use this site. An asterisk marks *Sph*I and *Bgl*II sites that were lost during cloning of the *′araD′* fragment. Arrows show gene and promoter orientations. *bla*, β-lactamase gene; *tetA* and *tetR*, tetracycline resistance and repressor genes, respectively; *oriT*, origin of transfer from RP4; $T1_4$ and $T1T2_2$, transcription terminators; *kan*, kanamycin resistance gene; *lacZ* (op), promoterless *lacZ* gene for construction of transcriptional, i.e., operon (op), fusions (17). See text.

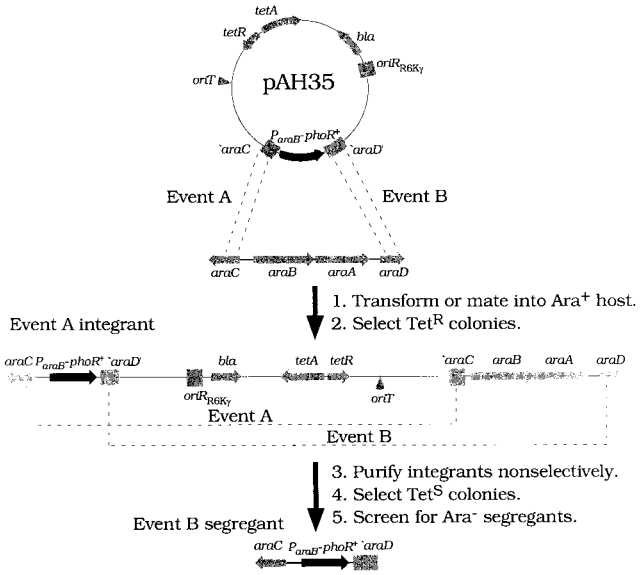

FIG. 3. Recombination of a P_{araB}-*phoR*$^+$ fusion onto the chromosome at the *araCBAD* locus. The construction of pAH35 is described in Table 2. pAH35 can integrate into the chromosome via either of two homologous recombination events (event A or event B). Only an event A integrant is shown for simplicity. Subsequent recombination events result in loss of the plasmid, regenerating the parental strain (event A) or an allele replacement (event B segregant). These are selectable on TSS agar; ones with an allele replacement are recognizable as Ara$^-$ colonies (see Materials and Methods). A Δ*phoR* mutant was used to recombine the P_{araB}-*phoR*$^+$ fusion onto the chromosome to avoid recombination at the *phoBR* locus; a wild-type strain can also be used. A black arrow shows the orientation of *phoR*. Grey arrows show the orientations of other genes. Grey boxes show *araC* or *araD* gene fragments and the *pir*-dependent vector origin (*ori*$R_{R6K\gamma}$). A prime indicates that a gene portion is missing on the side of the prime. An arrowhead marks the nick site of *oriT*, the RP4 conjugative transfer origin.

this for two reasons. First, we considered that such strains may be useful in our studies on protein-protein interactions between their gene products, as reported elsewhere (13). Second, we suspected that the deleterious effects of a Δ*phoU* mutation were in part due to high-level expression of the Pho regulon. Because expression of the *phoBR* operon is under positive autogenous control, high-level expression of the Pho regulon probably requires also high-level synthesis of PhoB and PhoR. We therefore expected to overcome these deleterious effects by down regulating PhoB or PhoR synthesis by using these foreign-regulated promoters.

Arabinose-independent expression of P_{araB} fusions. The above results show the P_{araB} promoter to be tightly regulated for expression of the *lacZ* structural gene. To assess whether P_{araB} is useful for tight control of a regulatory gene under similar conditions, we constructed strains carrying chromosomal P_{araB}-*phoB*$^+$ and P_{araB}-*phoR*$^+$ fusions. Each strain has also a precise deletion of the respective regulatory gene. They have in addition a deletion of *creC* (Δ*creBCD* or Δ*creABCD*) as well as genes for acetyl phosphate synthesis [Δ(*ackA pta*)], thus eliminating activation of PhoB by the kinase CreC or acetyl phosphate (33). We then examined PhoB- and PhoR-dependent control by measuring BAP levels under conditions of P_i limitation in the absence of arabinose during growth on different carbon sources. As shown in Table 5, P_{araB}-*phoB*$^+$ Δ*phoB* strain BW24803 exhibits a null phenotype in the absence of arabinose on all carbon sources. In contrast, P_{araB}-*phoR*$^+$ Δ*phoR* strain BW22861 exhibits a PhoR$^+$ phenotype in the absence of arabinose, even during growth on glucose. These results are consistent with less PhoR than PhoB being required for transcriptional activation of *phoA*. Therefore, P_{araB} appears to be sufficiently tightly controlled for the expression of *phoB*, but not for the expression of *phoR*. Yet, *phoR* expression is clearly limiting under these conditions, as much higher BAP levels are seen in the presence of arabinose (data not shown).

An eventual goal was to express both *phoB* and *phoR* independently and simultaneously from a foreign promoter(s). In order to find appropriate conditions to do this, we used strains with a deletion of the *pstSCAB-phoU* operon that leads to constitutive expression of the Pho regulon. Individual ones are also Δ*phoB* or Δ*phoR* as well as Δ*creC* and Δ(*ackA pta*), as described above. As shown in Table 6, *phoA* expression is abolished in the absence of PhoB or PhoR (compare BW24741 and BW24740 with BW24739). Introduction of a P_{phoB}-*phoB*$^+$ fusion in single copy elsewhere on the chromosome restores normal *phoA* expression (compare BW24770 with BW24739). Introduction of a P_{tac}-*phoB*$^+$ fusion in single copy restores *phoA* expression partially in the absence of IPTG and fully in the presence of IPTG (compare BW24775 without and with IPTG). This apparent leakiness of P_{tac} was expected, as proper regulation by LacI requires additional upstream and downstream operator sequences (20) that are absent in the P_{tac}-*phoB*$^+$ fusion. Yet partial inducer dependence is observed even for expression of a regulatory gene from P_{tac}.

Arabinose- and rhamnose-dependent expression of P_{araB}, P_{rhaB}, and P_{rhaS} fusions. We compared strains carrying chromosomal P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, P_{araB}-*phoR*$^+$, P_{rhaB}-*phoR*$^+$, and P_{rhaS}-*phoR*$^+$ fusions to determine which promoter fu-

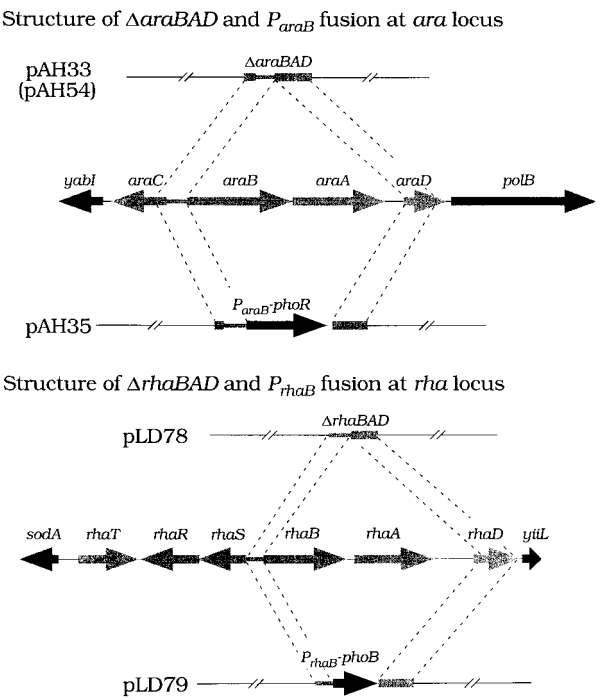

FIG. 4. Allele replacements at the chromosomal *araCBAD* and *rhaRSBAD* regions. (A) Structure of the Δ*araBAD* mutant and a P_{araB}-*phoR*$^+$ fusion at the *araCBAD* locus. (B) Structure of the Δ*rhaBAD* mutant and a P_{rhaB}-*phoB*$^+$ fusion at the *rhaRSBAD* locus. Black arrows represent P_{araB}-*phoR* and P_{rhaB}-*phoB* fusions. Light grey arrows signify genes belonging to the *ara* and *rha* gene clusters. Dark grey arrows show the *yabI* and *polB* genes flanking the *ara* locus and the *sodA* and *yiiL* genes flanking the *rha* locus. Dotted lines indicate regions where homologous recombination between the plasmid and the chromosome can occur. See text.

TABLE 4. Regulation of P_{araB}-, P_{rhaB}-, and P_{rhaS}-*lacZ* fusions

Strain	Relevant genotype[a]	Carbon source	β-Galactosidase sp act on[b]:		Fold induction
			−Ind	+Ind	
BW22831	P_{araB}-*lacZ rpoS*(Am)	D-Glucose	6.6 ± 0.1	1,270 ± 114	190
		D-Fructose	6.7 ± 0.6	3,861 ± 313	580
		Glycerol	6.5 ± 0.4	3,853 ± 119	590
BW22887	P_{rhaB}-*lacZ rpoS*(Am)	D-Glucose	0.5 ± 0.0	743 ± 44	1,600
		D-Fructose	0.5 ± 0.0	3,300 ± 67	6,900
		Glycerol	0.5 ± 0.0	3,689 ± 72	7,800
BW22886	P_{rhaB}-*lacZ rpoS*$^+$	D-Glucose	0.4 ± 0.0	185 ± 10	430
		D-Fructose	0.4 ± 0.0	1,158 ± 33	2,700
		Glycerol	0.4 ± 0.0	2,209 ± 50	5,800
BW22888	P_{rhaS}-*lacZ rpoS*$^+$	D-Glucose	0.4 ± 0.0	56 ± 2	130
		D-Fructose	0.5 ± 0.1	253 ± 5	490
		Glycerol	0.8 ± 0.1	619 ± 21	810

[a] All strains are also Δ*araBAD* or Δ*rhaBAD*, as appropriate; complete genotypes are given in Table 1.
[b] Cells were assayed after 18 h of growth in 0.06% glucose–, 0.06% fructose–, or 0.1% glycerol–MOPS–2 mM P_i without (−Ind) or with (+Ind) the inducer arabinose or rhamnose. Values are nanomoles of *o*-nitrophenol made per unit of cell culture optical density at 420 nm (means ± standard deviations). Strains were grown and assayed in triplicate.

sion(s) and growth conditions were appropriate for studying gene regulation in the Pho regulon. This was done with strains that are otherwise similar to ones described above. We examined PhoB- and PhoR-dependent control of *phoA* expression by measuring BAP levels when strains were grown on one of four carbon sources (glucose, mannitol, fructose, or glycerol), which were expected to result in different levels of catabolite repression (7). As shown in Table 7, strains carrying a P_{araB}-*phoB*$^+$ or P_{rhaB}-*phoB*$^+$ fusion express a null phenotype in the absence of inducer. The same ones synthesized ca. 12-fold to 2,400-fold more BAP in the presence of inducer, depending upon the carbon source and promoter. The relative expression levels correlate well with expectation in regards to catabolite repression. Catabolite repression is more severe with glucose than mannitol, more severe with mannitol than fructose, and more severe with fructose than glycerol. The BAP levels in these P_{araB}-*phoB*$^+$ and P_{rhaB}-*phoB*$^+$ fusion strains (ca. 280 to 490 U; BW24774, BW24777, and BW24773) (Table 7) during growth on glycerol are similar to that in an otherwise isogenic wild-type strain (ca. 375 U; BW24739) (Table 6). Strains with a P_{rhaB}-*phoR*$^+$ fusion at two chromosomal sites were examined; no difference between them was seen.

Of the three *phoR* fusion strains examined, only one (P_{rhaB}-*phoR*$^+$ fusion strain BW24768) showed a null phenotype in the absence of inducer. Substantial activation was apparent in both the P_{araB}-*phoR*$^+$ and P_{rhaS}-*phoR*$^+$ fusion strains (BW24508 and BW24858) in the absence of inducer even during growth on glucose, indicating that both P_{araB} and P_{rhaS} are somewhat leaky. Their basal levels of expression are also subject to catabolite repression, as they vary with the carbon source. Upon induction with rhamnose, the P_{rhaB}-*phoR*$^+$ fusion strain synthesized ca. 17-fold to 2,300-fold more BAP; these BAP levels also correlate well with the carbon source. The addition of the respective inducer resulted in increased BAP synthesis in the P_{araB}-*phoR*$^+$ and P_{rhaS}-*phoR*$^+$ fusion strains as well.

Use of foreign promoters to bypass growth defect due to a Δ*phoU* mutation. We showed above that we can modulate expression of the Pho regulon in our P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, and P_{rhaB}-*phoR*$^+$ fusion strains by growing them on different carbon sources in the presence of the respective inducer. We therefore tested whether these conditions can be used to overcome the deleterious effect of a Δ*phoU* mutation. Whereas a Δ*phoU* mutant such as BW17142 grows extremely poorly under all conditions tested, an otherwise isogenic Δ(*pstSCAB-phoU*) mutant such as BW17335 grows reasonably well (Table 8) (26). These strains have a kanamycin resistance cassette in place of *phoU* and *pstSCAB-phoU* sequences, respectively. A similar strain with an unmarked Δ*phoU* mutation (BW18897; Table 8) also shows a severe growth defect. Yet all these strains synthesize similar amounts of BAP. To determine whether modulating expression of *phoB* or *phoR* can be used to overcome the growth inhibition due to a Δ*phoU* mutation, we constructed otherwise isogenic Δ*phoU* and Δ(*pstSCAB-phoU*) strains with a Δ*phoB* and Δ*phoR* mutation carrying the respective fusions. These strains have an unmarked Δ*phoU* mutation, as each has a kanamycin resistance marker elsewhere (Table 1).

As shown in Table 8, our Δ*phoU* Δ*phoB*, Δ(*pstSCAB-phoU*) Δ*phoB*, Δ*phoU* Δ*phoR*, and Δ(*pstSCAB-phoU*) Δ*phoR* strains carrying a P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, or P_{rhaB}-*phoR*$^+$ fusion grow equally well in the absence or presence of the respective inducer. The P_{araB}-*phoB*$^+$ and P_{rhaB}-*phoB*$^+$ fusion strains (BW24774, BW24950, BW24773, and BW24949) show a null phenotype in the absence of inducer. Each synthesizes ca.

TABLE 5. Inducer-independent expression of P_{araB}-*phoB*$^+$ and P_{araB}-*phoR*$^+$ fusions

Strain	Relevant genotype[a]	BAP sp act on[b]:		
		D-Glucose	D-Fructose	Glycerol
BW24803	P_{araB}-*phoB*$^+$ Δ*phoB phoR*$^+$	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
BW22861	P_{araB}-*phoR*$^+$ *phoB*$^+$ Δ*phoR*	26.4 ± 4.9	47.7 ± 5.1	58.8 ± 4.4

[a] Strains are Δ*creABCD* Δ(*ackA pta*) Δ*araBAD*; complete genotypes are given in Table 1. The P_{araB}-*phoB*$^+$ fusion is in single copy at att_λ; the P_{araB}-*phoR*$^+$ fusion is in single copy at the *araCBAD* locus.
[b] Cells were assayed after 18 h of growth in 0.06% glucose–, 0.06% fructose–, or 0.1% glycerol–MOPS medium containing 0.1 mM P_i without arabinose. Values are nanomoles of *p*-nitrophenol made per unit of cell culture optical density at 420 nm (means ± standard deviations). Strains were grown and assayed in triplicate.

TABLE 6. Inducer-dependent control by using a P_{tac}-*phoB*$^+$ fusion

Strain	Relevant genotype[a]	BAP sp act on[b]:	
		−IPTG	+IPTG
BW24739	*phoBR*$^+$ Δ(*pstSCAB-phoU*)	376 ± 19	N.D.
BW24741	Δ*phoB phoR*$^+$ Δ(*pstSCAB-phoU*)	0.2 ± 0.0	N.D.
BW24740	*phoB*$^+$ Δ*phoR* Δ(*pstSCAB-phoU*)	0.2 ± 0.0	N.D.
BW24770	P_{phoB}-*phoB*$^+$ Δ*phoB phoR*$^+$ Δ(*pstSCAB-phoU*)	376 ± 17	N.D.
BW24775	P_{tac}-*phoB*$^+$ Δ*phoB phoR*$^+$ Δ(*pstSCAB-phoU*)	21.6 ± 0.9	355 ± 12

[a] All strains are *lacI*^{q} Δ*creABCD* Δ(*ackA pta*); complete genotypes are given in Table 1. The P_{phoB}-*phoB*$^+$ and P_{tac}-*phoB*$^+$ fusions are in single copy at att_λ.
[b] Cells were assayed after 18 h of growth in 0.06% glucose–MOPS–2 mM P_i without (−) or with (+) IPTG. BAP specific activity values are as defined for Table 5. N.D., not determined.

500-fold more BAP in the presence of the respective inducer. Likewise, the P_{rhaB}-*phoR*$^+$ fusion strains (BW24768 and BW24948) show a null phenotype in the absence of rhamnose; each synthesizes ca. 500-fold more BAP in its presence. Upon induction these strains synthesize ca. 25% of the normal amount of BAP (see BW24739 for comparison), suggesting that this level of Pho regulon gene expression is apparently insufficient to result in a severe growth defect due to a Δ*phoU* mutation.

DISCUSSION

Our primary goal was to develop a method(s) to study PhoU function. The severe growth defect of a Δ*phoU* mutant is especially problematic due to the rapid accumulation of compensatory mutants with lesions in *phoB*, *phoR*, or a *pst* gene. A Pst$^+$ Δ*phoU* mutant is also exquisitely sensitive to extracellular P_i, suggesting that PhoU has, in addition to its function in P_i signaling, a role as an enzyme in intracellular P_i metabolism (26). As shown here, we were able to overcome the deleterious effect of a Δ*phoU* mutation by uncoupling PhoB or PhoR synthesis from its normal autogenous control and expressing *phoB* or *phoR* from a foreign promoter(s). To do this, we constructed strains carrying a P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, P_{araB}-*phoR*$^+$, or P_{rhaB}-*phoR*$^+$ fusion on the chromosome. We used strains with single-copy chromosomal fusions to avoid problems resulting from the use of plasmids, such as an antibiotic requirement, variable plasmid copy number, and even plasmid loss. We also examined ways for modulating expression levels from the P_{araB} and P_{rhaB} promoters. We already reported work with similar strains and growth conditions to study the regulatory genes of the *Enterococcus faecium* vancomycin resistance gene cluster in an *E. coli* model system (12).

We constructed the corresponding P_{araB} and P_{rhaB} fusion strains with conditionally replicative (*pir*-dependent) allele replacement plasmids pAH33, pAH54, and pLD78 (Fig. 2). Strains carrying these fusions are made via a simple two-step procedure as illustrated in Fig. 3. The resulting P_{araB} and P_{rhaB} fusions reside on the chromosome at the *araCBAD* and *rhaRSBAD* loci, respectively, as shown in Fig. 4. We made similar strains with the respective Δ$araBAD_{AH33}$ and Δ$rhaBAD_{LD78}$ chromosomal mutations as controls. In these ways, we constructed a number of strains that express various normal or mutant *pho*, *cre*, or *van* genes under the control of P_{araB} or P_{rhaB} in single copy on the chromosome at the respective loci (12–14, 16).

The P_{araB} promoter (also called P_{BAD}) has been shown elsewhere to be tightly regulated (11, 21). In those studies, genes that are normally expressed at moderate levels were examined. In preliminary studies, we found that a pBAD plasmid (11) carrying *phoR* did not provide sufficiently tight control for our purposes, suggesting that these plasmids are somewhat leaky (14). This is consistent with *phoR* normally being expressed at a very low level. In order to reduce this basal level of expression, we recombined several P_{araB}-*phoR* fusions onto the chromosome by using derivatives of P_{araB} fusion allele replacement plasmid pAH33. When single-copy fusion strains were used,

TABLE 7. Inducer-dependent control by using P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, P_{araB}-*phoR*$^+$, P_{rhaB}-*phoR*$^+$, and P_{rhaS}-*phoR*$^+$ fusions

Strain	Relevant genotype[a]	Inducer[b]	BAP sp act on[c]:			
			D-Glucose	D-Mannitol	D-Fructose	Glycerol
BW24774	P_{araB}-*phoB*$^+$ Δ*phoB* Δ(*pstSCAB-phoU*)	None	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
		Ara	4.0 ± 0.4	103 ± 0	174 ± 6	281 ± 5
BW24777	P_{rhaB}-*phoB*$^+$ Δ*phoB* Δ(*pstSCAB-phoU*)	None	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
		Rha	2.6 ± 0.3	120 ± 23	350 ± 36	442 ± 5
BW24773	P_{rhaB}-*phoB*$^+$ Δ*phoB* Δ(*pstSCAB-phoU*)	None	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
		Rha	2.4 ± 0.3	104 ± 9	359 ± 11	486 ± 2
BW24508	P_{araB}-*phoR*$^+$ Δ*phoR* Δ(*pstSCAB-phoU*)	None	24.8 ± 0.5	36.3 ± 1.6	75.5 ± 7.4	118 ± 3
		Ara	149 ± 6	483 ± 9	336 ± 29	400 ± 27
BW24768	P_{rhaB}-*phoB*$^+$ Δ*phoR* Δ(*pstSCAB-phoU*)	None	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
		Rha	3.4 ± 0.8	108 ± 13	357 ± 4	464 ± 2
BW24858	P_{rhaS}-*phoR*$^+$ Δ*phoR* Δ(*pstSCAB-phoU*)	None	78.5 ± 5.4	85.7 ± 8.1	161 ± 11	257 ± 15
		Rha	92.4 ± 4.5	175 ± 13	398 ± 14	673 ± 5

[a] All strains are Δ*creABCD* Δ(*ackA pta*) and Δ*araBAD* or Δ*rhaBAD*, as appropriate; complete genotypes are given in Table 1. The P_{araB}-*phoB*$^+$ and P_{rhaB}-*phoB*$^+$ fusions are in single copy at att_λ; the P_{rhaB}-*phoB*$^+$ and P_{araB}-*phoR*$^+$ fusions are in single copy at the *rha* and *ara* loci, respectively; the P_{rhaB}-*phoR*$^+$ and P_{rhaS}-*phoR*$^+$ fusions are in single copy at att_{HK022}.
[b] Cells were assayed after 18 h of growth in 0.06% glucose–, 0.06% mannitol–, 0.06% fructose–, or 0.1% glycerol–MOPS medium containing 2 mM P_i without (none) or with arabinose (Ara) or rhamnose (Rha) for induction.
[c] BAP specific activity values are as defined for Table 5.

TABLE 8. Pho regulon control in Δ*phoU* mutants by using P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, and P_{rhaB}-*phoR*$^+$ fusions

Strain	Relevant genotype[a]	Growth[b]	BAP sp act on[c]:	
			−Ind	+Ind
BW17142	Δ*phoU*	+/−	419[d]	N.D.
BW17335	Δ(*pstSCAB-phoU*)	+++	428[d]	N.D.
BW18897	Δ*phoU*	+	444 ± 33	N.D.
BW24739	Δ(*pstSCAB-phoU*) Δ(*ackA pta*)	+++	443 ± 20	N.D.
BW24774	P_{araB}-*phoB*$^+$ Δ*phoB* Δ(*pstSCAB-phoU*) Δ(*ackA pta*)	++++	0.2 ± 0.0	94.8 ± 3.5
BW24950	P_{araB}-*phoB*$^+$ Δ*phoB* Δ*phoU* Δ(*ackA pta*)	++++	0.2 ± 0.0	113 ± 3
BW24773	P_{rhaB}-*phoB*$^+$ Δ*phoB* Δ(*pstSCAB-phoU*) Δ(*ackA pta*)	++++	0.2 ± 0.0	109 ± 27
BW24949	P_{rhaB}-*phoB*$^+$ Δ*phoB* Δ*phoU* Δ(*ackA pta*)	++++	0.2 ± 0.0	89.7 ± 6.4
BW24768	P_{rhaB}-*phoR*$^+$ Δ*phoR* Δ(*pstSCAB-phoU*) Δ(*ackA pta*)	++++	0.2 ± 0.0	104 ± 2
BW24948	P_{rhaB}-*phoR*$^+$ Δ*phoR* Δ*phoU* Δ(*ackA pta*)	++++	0.2 ± 0.0	83.4 ± 8.9

[a] Complete genotypes are given in Table 1. The P_{araB}-*phoB*$^+$ and P_{rhaB}-*phoB*$^+$ fusions are in single copy at att_λ; the P_{rhaB}-*phoR*$^+$ fusion is in single copy at att_{HK022}.
[b] Colony size after 24 h of incubation +/−, barely detectable; +, tiny; +++, ca. 1 mm in diameter; ++++, ca. 1.5 mm in diameter.
[c] Cells were assayed after 18 h of growth in 0.06% mannitol–MOPS–2 mM P_i without (−Ind) or with (+Ind) arabinose or rhamnose for induction. BAP specific activity values are as defined for Table 5. N.D., not determined.
[d] BAP specific activity value is an average of four independent determinations (unpublished data cited in reference 26).

particular P_{araB}-*phoR* fusions, e.g., one expressing only the C-terminal kinase domain of PhoR (13), no longer appeared to be leaky. Yet a single-copy P_{araB}-*phoR*$^+$ fusion strain synthesizes sufficient PhoR for (partial) activation in the absence of arabinose even in the presence of glucose (Table 5). These results suggest that full-length PhoR is a more active kinase than its C-terminal domain. However, in the absence of arabinose, the amount of PhoR synthesis from a P_{araB}-*phoR*$^+$ fusion is also clearly limiting because upon P_i limitation only ca. 10% as much BAP is made in this strain as in a wild-type strain. In contrast, a single-copy P_{araB}-*phoB*$^+$ fusion strain shows no leakiness as it exhibits a PhoB$^-$ phenotype in the absence of inducer on all carbon sources tested (Table 7). Therefore, more PhoB than PhoR is apparently required for expression of the Pho regulon. These data are consistent with PhoR acting catalytically as an autokinase and phosphotransferase in the activation (phosphorylation) of PhoB, which in turn acts as a transcription factor.

In order to control PhoB and PhoR synthesis independently, we also constructed strains with a P_{rhaB}-*phoB*$^+$ or P_{rhaB}-*phoR*$^+$ fusion. Although both L-arabinose and L-rhamnose act directly as inducers for expression of regulons for their catabolism, important differences exist in regard to the regulatory mechanisms (Fig. 5). L-Arabinose acts as inducer with the activator AraC in the positive control of the arabinose regulon (23). However, the L-rhamnose regulon is subject to a regulatory cascade; it is therefore subject to an even tighter control. L-Rhamnose acts as an inducer with the activator RhaR for synthesis of RhaS, which in turn acts as an activator in the positive control of the rhamnose regulon (6). As shown in Table 7, both the P_{rhaB}-*phoB*$^+$ and P_{rhaB}-*phoR*$^+$ fusion strains show a PhoB$^-$ and PhoR$^-$ phenotype, respectively, in the absence of rhamnose. We also examined a P_{rhaS}-*phoR*$^+$ fusion. Like the P_{araB}-*phoR*$^+$ fusion strain, the P_{rhaS}-*phoR*$^+$ fusion strain synthesized a substantial, though limited, amount of BAP in the absence of induction.

The L-arabinose and L-rhamnose regulons are also regulated by catabolite repression. We therefore modulated expression of P_{araB}, P_{rhaB}, and P_{rhaS} fusions by using various carbon sources (glucose, mannitol, fructose, and glycerol) that lead to different levels of catabolite repression (7). When strains are grown on these carbon sources with the inducer in excess, the expression of the Pho regulon can be modulated 70- to 200-fold in our P_{araB}-*phoB*$^+$, P_{rhaB}-*phoB*$^+$, and P_{rhaB}-*phoR*$^+$ fusion strains (Table 7). As expected, the differences are much smaller in the P_{araB}-*phoR*$^+$ and P_{rhaS}-*phoR*$^+$ fusion strains as the expression of these fusions is also leaky under these conditions. In preliminary experiments, we had also attempted to modulate expression levels by using different inducer concentrations. However, we were unable to maintain a constant, steady state level of induction (data not shown) (5). An inability to maintain steady-state induction at intermediate levels by the limiting inducer concentration is expected as the presence of arabinose and rhamnose results also in increased synthesis of their respective transport systems (Fig. 5). This has now been substantiated by monitoring P_{araB} expression levels in cells grown in the presence of subsaturating inducer concentrations (24). Importantly, as shown here, expression of these promoters can be modulated over a wide range by using different carbon sources in the presence of saturating inducer concentrations.

Both PhoU and the Pst transporter have been implicated in the negative control of the Pho regulon, as mutations of either result in high constitutive Pho regulon gene expression. Pre-

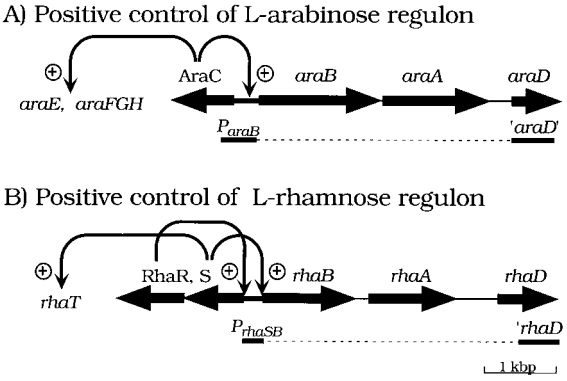

FIG. 5. Transcriptional activation of the arabinose and rhamnose regulons. (A) Positive control of the L-arabinose regulon. The *araCBAD* region near 1.5 min on the *E. coli* chromosome is shown. The unlinked *araE* and *araFGH* genes encode arabinose transport systems (23). (B) Positive control of the L-rhamnose regulon. The *rhaRSBAD* region near 88.2 min is shown. The nearby *rhaT* gene is transcribed towards *rhaR* (Fig. 4) and encodes a rhamnose permease (3). Thick arrows show gene orientations. Curved ones with circled pluses indicate sites for transcriptional activation by AraC (A) or RhaR and RhaS (B). The lower bars show the *ara* and *rha* sequences in the respective P_{araB} and P_{rhaB} fusion allele replacement vectors illustrated in Fig. 2. The dashed lines show the chromosomal regions deleted in the resultant recombinants. See text.

sumably, they somehow act together. To study how they interact with the PhoB-PhoR system, we made strains that synthesize PhoB or PhoR under control of the tightly regulated P_{araB} and P_{rhaB} promoters. By using these strains, we were able to express *phoB* or *phoR* at a reduced level and thereby overcome the harmful effect of a Δ*phoU* mutation. In these ways, it should now be possible to study further the function(s) of PhoU.

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