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The *phoBR* Operon in *Escherichia coli* K-12

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The *phoB* and *phoR* genes encode a transcription activator and a sensory protein of the phosphate regulon, respectively. It is shown here that they were transcribed as an operon in which the *phoB* gene was promoter proximal. Although an operon structure was suggested previously (K. Makino, H. Shinagawa, M. Amemura, and A. Nakata, J. Mol. Biol. 190:37–44 and 192:549–556, 1986), previous results showed only that *phoR* gene expression during phosphate limitation is dependent on the upstream *phoB* promoter. The *phoR* gene could still have had its own promoter for expression in the presence of phosphate. Two polar transposon-induced mutations are described which simultaneously abolished *phoB* and *phoR* gene function in *cis*; one mutation mapped in the *phoB* gene, and the other mapped upstream of the *phoB* gene. These results demonstrate an operon structure, in which *phoR* gene function required expression from the *phoB* promoter. Unexpectedly, an antisense *phoΩ*Mu d1(*lacZ*) insertion within the promoter-proximal end of the *phoB* gene expressed the *lacZ* reporter gene, thus allowing for the possibility that the *phoBR* operon is regulated by an antisense RNA.

The phosphate regulon consists of more than 20 promoters that are regulated by the availability of phosphate in the growth medium. When the P_i levels become growth limiting, the synthesis of about 90 phosphate-starvation-inducible (Psi) proteins is induced (F. C. Neidhardt, T. Phillips, and B. L. Wanner, unpublished results). One set of Psi proteins is encoded by five unlinked genes and operons whose products allow for the more efficient transport of P_i or the utilization of alternative phosphorus sources (23, 27). The transcription of the *psi* genes involved in phosphorus assimilation is regulated by the PhoB and PhoR proteins (27). The prototypic Psi protein is bacterial alkaline phosphatase (BAP), which is the *phoA* gene product.

The *phoB* gene encodes a transcriptional activator; the *phoR* gene encodes a phosphate-sensory protein (27). The expression of the *phoB* gene itself is both autogenously controlled (9) and phosphate regulated (9, 19). This is consistent with the *phoB* promoter having a consensus sequence overlapping its −35 promoter region, which is called the phosphate box and is shared with other *phoB*-dependent *psi* promoters (13). The *phoB* and *phoR* genes map at 9 min on the *Escherichia coli* chromosome. The *phoB* gene is transcribed clockwise, i.e., towards the *phoR* gene (9); and the presumed translation start codon for the PhoR protein lies 61 base pairs downstream from the translation stop codon for the *phoB* structural gene (14).

The PhoR protein has a dual regulatory role as both an activator and a repressor (28). By constructing a *phoR-lacZ* transcriptional fusion in vitro and studying its expression on a plasmid, Makino et al. (15) found that *phoR* gene expression is also induced by phosphate limitation; this induction requires the *phoB* promoter region. These results led to the conclusion that the *phoB* and *phoR* genes are in an operon in which the *phoR* gene is expressed from the *phoB* promoter. However, a basal level of *phoR-lacZ* transcription is seen in constructs lacking the *phoB* promoter or having a transcription terminator inserted in the *phoB* gene. Also, similar constructs with a $phoR^+$ gene in place of the fusion fully complement *phoR* mutants for repression and almost fully complement them for transcriptional activation of the *phoA* gene (15).

One possibility that could account for the expression of the *phoR* gene in the absence of the *phoB* promoter would be the existence of multiple promoters; the *phoR* gene could be expressed at a low level from its own promoter in the presence of phosphate and expressed at a higher level from the *phoB* promoter only during phosphate limitation. This interpretation is entirely consistent with the results described previously (15). Also, workers in another laboratory (21) previously concluded that the *phoB* and *phoR* genes are not in an operon, a conclusion reached because several Tn*5* insertions within the *phoB* gene fail to abolish *phoR* gene function. Although the Tn*5* insertions were isolated on a high-copy-number plasmid, which could have complicated the results, a chromosomally located *phoBΩ*Tn*5* insertion was also nonpolar, i.e., the mutant was phenotypically $PhoB^-$ and $PhoR^+$ (this laboratory, unpublished data).

Two chromosomal *phoΩ*Mu d1 mutants are characterized here. They were identified as *phoB* negative; both are polar, and they simultaneously abolished *phoR* gene function in *cis*. One mutation mapped within the *phoB* gene; the other mapped upstream of the *phoB* gene, presumably within the *phoB* promoter region. The total loss of both *phoR* gene functions, i.e., transcriptional activation and repression, shows that the *phoB* and *phoR* genes were transcribed solely as an operon from a promoter(s) upstream of the *phoB* structural gene. A $pho(BR)^+$ transducing bacteriophage is also described.

MATERIALS AND METHODS

Strains and methods. The φ80 d13(*proC*$^+$ *phoB*$^+$ *phoR'*) lysates were made by UV induction of a strain XPh43 dilysogen (31). φ80 d13 lysogens were selected as Pro^+ transductants of $\phi80^+$ lysogens that were $ProC^-$. The φ80p1 *phoA*$^+$ phage and its *pho-1003*(Bin) (Bin, *phoB*-independent expression of the *phoA* gene) recombinant were described elsewhere (30). The *phoA*$^+$(Bin) phage has a promoter mutation which renders BAP expression *phoB* independent (12, 30). M13mp10 and mp11 (16) were provided by A. Shauer, and p1088 (*int*$^+$) was from S. Garrett. λB55 [*imm*^{21c} Δ(*int-red*)*h*80], λB476 [*imm*^{21c} Δ(*int-red*)*h*λ], λB107 (*imm*^{21c} *b*2), and λY2 (*c*I857 *b*2) are from S. Mizusawa. λB55 and

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λB476 were used to test for phage 21 immunity, and λB107 or λY2 was used to supply the Int protein. Strain LE292 was from L. Enquist and was used as previously described (8). Other strains are from laboratory stocks (24, 26). The chromogenic substrates (blue dyes) XP and XG were used to detect BAP and β-galactosidase activities (30), respectively. Recombinant DNA methods were described elsewhere (9, 26). Mapping phage lysates were made on an appropriate Δ*phoA* or Δ*phoB* mutant, which was necessary to reduce BAP enzyme activity in the lysates.

Isolation of *pho*::Mu d1 mutants. Mu d1 donor lysates were prepared by heat induction of strain MAL103 (4) and used to transduce strain BW646 (24; Δ*lac-169 proC*::Tn*5 ilv-691*::Tn*10 aroB rpsL267 crp-72 pho-510 thi*) to ampicillin resistance. Transductants were selected at 30°C on ampicillin agar that was based on TYE-XP, TYE-LPM-XP (a rich medium with a reduced phosphate concentration), and glucose MOPS (morpholinepropanesulfonic acid)-XP media (30) with 2 mM or 0.1 mM K_2HPO_4 and required supplements. Among about 20,000 colonies, 850 transductants showed an altered BAP phenotype. Of these, only about 15% behaved as BAP structural gene or regulatory mutants upon further testing. Independent mutants were characterized phenotypically and by mapping and then stored frozen (24) as strains BW1600 to BW1698 and BW1731 to BW1738 (31). The mutants were converted to λp1(209) lysogens as described previously (29). Replacing the Mu d1 sequences with λp1(209) sequences eliminates Mu sequences that are required for efficient transposition. Hence, λp1(209) lysogens are relatively more stable and useful in subsequent genetic studies (29).

Identification of a plaque-forming *pho(BR)*$^+$ transducing phage. λBW341 is a *pho(BR)*$^+$ λD69 recombinant phage; it was identified in a library made by S. Mizusawa and D. Ward and corresponds to the *phoR*$^+$ phage in Table IV of reference 18. It has a partially digested *Sau*3A fragment of *Escherichia coli* SM57 DNA in the *Bam*HI site, within the *int* gene, of λD69 (18; S. Mizusawa, personal communication). λBW341 was identified as follows. (i) Dilutions of the λD69 library were preadsorbed to strain BW322 (*phoB452 phoR68*; 29), and 10^8 cells were then plated in 3 ml of H top agar with 25 μl of XP (40 mg/ml in dimethylformamide) onto glucose MOPS agar lacking phosphate. (ii) Among several thousand white plaques, four dark blue ones were found, and the plaques were purified on a lawn of strain BW322. (iii) Lysogens were made, and one that was phenotypically PhoB$^+$ and PhoR$^+$ was UV induced. The phage plaques were purified on a lawn of strain BW3212 [Δ(*phoBR*)*9-6*; 24], and the phage was named λBW341. Although λBW341 complemented all *phoB* and *phoR* mutations tested, it failed to complement mutations in the *phoA*, *proC*, *aroLM*, *tsx*, or *nusB* genes that map nearby (24; data not shown). Bacteria were lysogenized with λBW341 by supplying an *int*$^+$ gene in *trans*. Int function was provided with either an *int*$^+$ helper phage or the p1088 plasmid. The latter was introduced by DNA transformation or conjugal transfer, since p1088 is a Mob$^+$ plasmid. Plasmid transfer was done from strain BW358(p1088) in tripartite matings with pRK2013 as a helper plasmid, as described previously (26).

Construction of M13 mapping phages. λBW341 and pUC12 DNAs were cut with *Eco*RI, mixed, and ligated to construct *pho(BR)*$^+$ plasmids, which also contained some λD69 DNA. The plasmid pBC9 has the *phoB* gene in the same orientation as the *lac* promoter in pUC12; pBC6 has the *phoB* gene in the opposite orientation. Plasmid pBC6 DNA was digested with *Eco*RI, and its 7.2-kilobase (kb) *pho(BR)*$^+$ insert was purified on agarose, electroluted, digested with *Sau*3A, and ligated with *Bam*HI-cut M13mp10 and mp11 phages. The *Sau*3A clones with *phoB* DNA fragments were identified by marker rescue with a reference set of *phoB* mutants and further characterized by the C test (32). M13mp recombinant phages with 0.3- or 0.6-kb inserts in both orientations rescued various *phoB* mutations that were made with chemical or *mutD* mutagenesis (unpublished data).

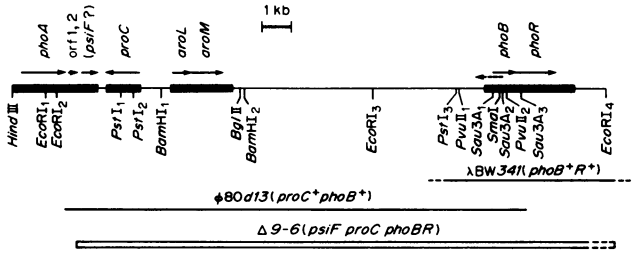

FIG. 1. Physical structure of the *phoA-proC-aroLM-phoBR* region. The *phoA-orf-1-orf-2* (5, 20), *proC* (7), *aroLM* (6), *phoB* (13), and *phoR* (14) DNAs that were sequenced are indicated by the solid bars. The *orf-1* or *orf-2* DNA may be the *psiF* (31) structural gene, whose promoter is phosphate starvation inducible (31). As noted previously (11), the *proC* DNA sequence is partially incorrect. Only reference restriction sites are shown which were either reported elsewhere (1, 9, 10, 21, 22) or determined here. The *Sau*3A$_1$ site is at −34 base pairs, and *Sau*3A$_2$ is at +266 base pairs with respect to the translation initiation codon for the *phoB* gene (13). The *Sau*3A$_3$ site is within the amino end of the *phoR* gene (14). In the text, the *Sau*3A$_1$-*Sau*3A$_2$ and *Sau*3A$_2$-*Sau*3A$_3$ fragments are called the 0.3- and 0.6-kb fragments, respectively. The chromosomal DNAs in the λBW341 and ϕ80 d13 phages are shown below the map. ϕ80 d13 carries the extreme carboxyl end of the *phoA* gene because it rescues promoter-distal *phoA* mutations (unpublished results). It also carries 0.4 to 0.5 kb for the 5′ end of the *phoR* gene, in agreement with the restriction map of the *phoB*$^+$ plasmid pHI4 that was made from this phage (9). The ϕ80 d13 phage probably has the *phoR68* allele because it was isolated from a *phoR68* mutant (2) and heterozygous ϕ80 d13 *pho(BR)*$^+$ lysogens segregate constitutive *phoR* homogenotes (unpublished results). Therefore, the *phoR68* allele probably maps within the 5′ end of the *phoR* gene. The Δ 9-6 mutation removes the *psiF-phoR* DNA interval and was described elsewhere (31). It was characterized both genetically and by Southern hybridizations (data not shown). The *spcC* gene also maps in the *proC-phoBR* interval (12a), but its precise location is unknown. The broken lines indicate regions which are less well defined. The arrows show the directions of transcripts.

Two other M13mp mapping phages were also used here. Both contained a similar DNA fragment which included the 5′ end of the *phoB* gene plus about 1 kb of DNA upstream. An M13mp phage with the *Pst*I$_3$-*Sma*I fragment (Fig. 1) was made by digesting the plasmid pBC9 with *Sma*I and religating it. This removed the carboxyl end of the *phoB* gene, whose *Sma*I site was now joined to the polylinker. The 5′ end fragment of the *phoB* gene was subcloned from this deleted plasmid as a *Hin*dIII fragment by using the polylinker *Hin*dIII site and a second *Hin*dIII site that originated from λ DNA and mapped upstream of the *phoB* gene on the plasmid. The *Hin*dIII fragment was cloned into M13mp11, and a chimeric phage with the *phoB* DNA fragment in the opposite orientation to the *lac* promoter was identified. This phage was digested with *Pst*I and religated to construct a mapping phage with the *Pst*I$_3$-*Sma*I DNA fragment.

Restriction mapping revealed that the *Pst*I$_3$-*Sma*I mapping phage had a useful *Pvu*II site within the insert (*Pvu*II$_1$ in Fig. 1), which was very close to the *Pst*I$_3$ site. A *Pvu*II$_2$ site also

TABLE 1. Complementation of *phoBR* mutants by *phoB*$^+$ and *pho(BR)*$^+$ transducing phages[a]

Chromosome	Phage	BAP sp act (U per OD_{420})		β-Galactosidase sp act (U per OD_{420})	
		+P_i	−P_i	+P_i	−P_i
Wild type	None	1.7	243	ND	ND
Δ(*phoBR*)*9-6*	None	0.2	0.2	ND	ND
Δ *9-6*	φ80 d13(*phoB*$^+$)	35.8	33.5	ND	ND
Δ *9-6*	λBW341 [*pho(BR)*$^+$]	1.5	197	<0.2	<0.2
phoBΩ87	None	0.2	0.8	0.9	0.6
phoBΩ87	φ80 d13(*phoB*$^+$)	31.2	35.5	0.5	0.5
phoBΩ87	λBW341 [*pho(BR)*$^+$]	0.2	193	0.4	0.6
phoBΩ766	None	0.3	0.9	<0.2	<0.2
phoBΩ766	λBW341 [*pho*(BR)$^+$]	0.9	101	<0.2	<0.2
phoBΩ766	φ80 d13(*phoB*$^+$)	27.6	32.3	<0.2	<0.2

[a] Strains BW646, BW1965, BW2350, BW5943, BW1616, BW5916, BW5893, BW1667, BW5831, and BW5917 were grown in 0.4% glucose-MOPS medium with 0.1 mM K_2HPO_4 plus required supplements. All mutants are Δ*lac-169 pho-510 rpsL267 aroB thi* strains; the *phoBΩ87* and *phoBΩ766* mutants also have a *proC*::Tn*5* mutation. Samples were removed during the logarithmic growth phase (+P_i) and 2 to 6 h thereafter (−P_i) and assayed as described elsewhere (27, 29). Units are nanomoles of product made per minute per culture optical density at 420 nm. Average values of three or more determinations are given. ND, Not determined.

was found in the *phoB* gene (Fig. 1). An M13 mapping phage with the amino-terminal end of the *phoB* gene and its upstream region was constructed with the *PvuII*$_1$-*PvuII*$_2$ fragment. To do this, plasmid pBC6 was digested with *PstI* and religated to remove DNA to the left of the *PstI*$_3$ site in Fig. 1. The deleted plasmid was cut with *PvuII*, mixed with *Sma*I-cut M13mp11 replicative form DNA, and ligated. Phage with the amino-terminal *PvuII phoB* fragment were identified by marker rescue and verified with the C test by using the appropriate M13mp *Sau*3A clones described above.

Marker rescue experiments with M13 mapping phage. The mapping of *phoB* mutations was done by spotting M13 phage onto a lawn of a *recA*$^+$ *phoB* mutant with an F′ *lacI*(Q*1*) episome. Selection was for growth on glucose MOPS-XP agar lacking P_i, i.e., for the utilization of XP as a sole phosphorus source. Rescuable mutants gave dark blue colonies. Representative recombinants were purified and tested for restoration of the PhoR$^+$ character. In the case of the *phoΩ87*:: and *phoΩ766*::λp1(209) mutants, recombinants were also tested for loss of λ immunity to verify that true recombinants were selected.

RESULTS

Characterization of the *phoΩ87*:: and *phoΩ766*::Mu d1 mutants. Two Mu d1-induced mutations are used here to show that the *phoB* and *phoR* genes were transcribed as an operon. The mutations were identified in strains BW1616 (*phoΩ87*) and BW1667 (*phoΩ766*), which were isolated as BAP-negative mutants following Mu d1 mutagenesis of strain BW646. Both mapped near the *proC* gene (data not shown), and their BAP-negative phenotype was complemented with the φ80 d13(*phoB*$^+$) and φ80p1 *phoA*$^+$(Bin) phages but not with the φ80p1 *phoA*$^+$ phage, suggesting that the mutants have *phoB*::Mu d1 insertions. The mutants differed in two ways, however. The *phoΩ766* mutant appeared to have a leaky mutation, which is unexpected for a Mu d1 insertion within a structural gene; the *phoΩ87* mutant was completely BAP negative. (*phoB* mutants are generally white [BAP negative] on low-phosphate XP agar; the *phoΩ87* mutant was white and the *phoΩ766* mutant was distinctly blue.) Neither made a significant amount of enzyme is standard BAP assays (Table 1, rows 5 and 8).

These mutants also differed with respect to their Lac phenotype; strain BW1616 (*phoΩ87*) was Lac$^+$ (pale blue) on XG agar, while strain BW1667 (*phoΩ766*) was Lac$^-$ (white). Both mutants were Lac$^-$ on MacConkey or lactose minimal agar. The Lac$^+$ phenotype indicates that the Mu d1(*lacZ*) gene was fused to a transcriptional signal in the *phoΩ87* mutant; a Lac$^-$ character indicates the absence of a transcriptional fusion. Alternatively, the *lacZ* DNA could have been deleted (somehow) in the construction of the *phoΩ766* mutation. To show that both mutants contained the Mu d1 (*lacZY*) DNA, Lac$^+$ mutants were selected on lactose minimal agar. (Lac$^+$ derivatives of Lac$^-$ Mu d1 mutants arose frequently, presumably due to transposition of Mu d1.) Both strains yielded a similar frequency of Lac$^+$ mutants on lactose minimal agar, thus showing that the Mu d1(*lacZ*) transposon was probably intact.

Complementation tests. When the *phoΩ87* and *phoΩ766* mutants were tested in complementation experiments, the results clearly showed that both mutations simultaneously abolished *phoB* and *phoR* gene function in *cis*. When either was complemented by lysogenization with the φ80 d13 (*phoB*$^+$) phage, the lysogens were not only BAP positive but also BAP constitutive, i.e., the mutants were phenotypically both PhoB$^-$ and PhoR$^-$. Also, the complemented mutants made a reduced amount of enzyme even when phosphate starved (Table 1, lines 6 and 10). The reduced amounts of enzyme made during phosphate limitation are consistent with the loss of the PhoR protein as an activator (28). (In these strains, BAP synthesis in the absence of *phoR* gene function is dependent on the unlinked *pho-510* mutant form of the *phoM* operon [25].)

Additional complementation tests were done with F′ 13 [*pho(BR)*$^+$], F′ 13 *phoB*$^+$*R70*(Am), F′ 13 *phoB*$^+$*R69*, λBW341 (*phoB*$^+$*R*$^+$), λBW341 *phoB513*(Am)*R*$^+$, and two λBW341 *phoB*$^+$ *phoR* mutant phages (data not shown). (The construction of these and other *phoB* and *phoR* mutant phages will be described elsewhere [manuscript in preparation].) In addition, all *pho(BR)*$^+$ DNAs restored a BAP-positive and phosphate-repressible BAP phenotype, *phoB phoR*$^+$ mutant DNAs failed to complement, and *phoB*$^+$ *phoR* mutant DNAs gave a BAP-positive and -constitutive phenotype. Furthermore, when an amber suppressor was crossed into heterozygous diploids that were made with a *phoB513*(Am) *phoR*$^+$ or a *phoB*$^+$ *phoR70*(Am) λBW341 phage, a BAP-positive and phosphate-repressible phenotype was restored. Phenotypically, both mutants behaved like a Δ(*phoBR*) mutant (Table 1). However, as shown below, both Mu d1 mutants had simple insertions, because they simultaneously reverted to a BAP-positive and phosphate-repressible character with loss of the Mu d1 DNA insert.

Reversion tests. The results presented above could be explained if the *phoB* and *phoR* genes were in an operon in which the *phoB* gene was promoter proximal. In this case, the *phoR* gene function would be abolished due to polarity. However, this seemed unlikely because a chromosomal *phoB*ΩTn*5* mutation was nonpolar; instead, a *phoB*ΩTn*5* mutant was BAP positive and phosphate repressible when complemented with the φ80 d13(*phoB*$^+$) phage (unpublished data). We thought, therefore, that the *phoΩ87* and *phoΩ766* mutants might have a deletion of the *phoR* gene that maps

nearby. The following tests ruled out this deletion hypothesis.

Although Mu-induced mutants do not yield precise excisions in a single step, Mu X$^-$ derivatives (3) do give rise to true revertants, and Mu X$^-$ mutants can be selected as temperature-resistant mutants of Mu d1 strains, which are Ts (temperature sensitive). To test for deletions, temperature-resistant mutants of the *phoΩ87* and *phoΩ766*::Mu d1 mutants were selected; BAP-positive revertants of these were selected for growth on glucose MOPS-XP agar lacking P_i. Both mutants gave numerous independent revertants in which the BAP-positive character was associated with loss of the Mu d1 prophage, as determined by testing for ampicillin resistance or by the ability to mutate to a Lac$^+$ phenotype. In these revertants, BAP synthesis was again phosphate repressible, thus showing that the *phoB* and *phoR* gene functions were simultaneously restored. This shows that the mutants have simple insertions. Curiously, some BAP-positive revertants of the *phoΩ766* mutant (but not of the *phoΩ87* mutant), which lost Mu d1, made reduced amounts of enzyme, suggesting that imprecise excision of Mu d1 in this mutant could partially restore a *phoB*$^+$ character (data not shown). Since BAP synthesis in these mutants was also repressed by phosphate, *phoR* gene function must have been simultaneously restored in them also. These results substantiate further the hypothesis that the *phoB* and *phoR* genes constitute an operon in which the *phoB* gene is promoter proximal. An imprecise excision in the *phoΩ766* mutant could partially restore *phoBR* operon function if the insertion maps upstream of the *phoB* structural gene.

Mapping the *phoΩ87* and *phoΩ766*::Mu d1 mutations. M13mp chimeric phages with portions of the *phoB* gene were used to map the mutations by marker rescue, as described in Materials and Methods. The *phoΩ87* mutation was rescued by the 0.3-kb *Sau*3A$_1$-*Sau*3A$_2$ fragment at the 5′ end of the *phoB* gene. It was also rescued by phages with the *Pst*I$_3$-*Sma*I fragment or the *Pvu*II$_1$-*Pvu*II$_2$ fragment containing the 5′ end of *phoB* and upstream sequences (Fig. 1). The *phoΩ87*::Mu d1 insertion, therefore, mapped between the *Sau*3A$_1$ and *Sma*I sites, which placed it either in the 5′ end of the *phoB* gene or in the 34 base pairs upstream of the *phoB* gene. Neither the 5′ nor the 3′ *phoB Sau*3A fragment rescued the *phoΩ766* mutation, however. Since it was rescued by the promoter-proximal *Pst*I$_3$-*Sma*I and *Pvu*II$_1$-*Pvu*II$_2$ fragments, which carry both the 5′ end of the *phoB* gene and its upstream region, the *phoΩ766* mutation probably maps upstream, i.e., within the *phoB* promoter region. Alternatively, it could map within the *phoB* gene and fail to yield recombinants because it maps very near to the *Sau*3A$_1$ site, which itself maps 34 base pairs upstream of the translated region (Fig. 1). However, a location in the upstream region is also consistent with an apparent leakiness of the *phoΩ766* mutation, as mentioned above.

Determining the orientation of the *phoΩ*Mu d1(*lacZY*) transposon insertions. The orientation of Mu d1 (*lacZ*) DNA in the mutants was determined by two methods; both procedures showed that the *lacZ* DNA was oriented in the counterclockwise direction on the chromosome, i.e., in the opposite direction with respect to *phoB* gene transcription. In one experiment, an F′ *lacZY* episome was integrated into the Mu d1(*lacZ*) DNA via homologous recombination, and the orientation of *lacZ* DNA was inferred by the direction of transfer of the respective F′ 42-*114*(Ts) Lac$^+$ integrant. To do this, strains BW3787 [*phoAΩ4*::λp1(209) (*lacZY*)], BW5998 [*phoΩ87*::λp1(209) (*lacZY*)], and BW5999 [*phoΩ766*::λp1(209) (*lacZY*)] were mated with the F′ 42-*114*(Ts$^-$) strain ECO (30) and Lac$^+$ exconjugants were identified with the recipient markers. Temperature resistant Lac$^+$ integrants were selected at 43°C and then mated with the multiply auxotrophic strain AB1157 (λ$^+$). [A λ$^+$ lysogen was used because the structure of F′ 42 is such that integration by *lac* homology leads to early transfer of the λp1(209) prophage.] When streptomycin-resistant exconjugants were selected, the number of prototrophic recombinants decreased in a counterclockwise order for the markers: Pro$^+$, Leu$^+$, Thr$^+$, Arg$^+$, and His$^+$. With either *phoB* mutant as donor, the number of Pro$^+$, Leu$^+$, and Thr$^+$ exconjugants exceeded the number of Arg$^+$ and His$^+$ recombinants by more than 100-fold. These data implied that both the *phoΩ87* and *phoΩ766* (*lacZY*) insertions had *lacZY* DNA in a counterclockwise orientation. However, the *phoAΩ4* mutant, which maps nearby and was used as a control, gave the highest number for His$^+$ exconjugants, thus showing a clockwise orientation, as expected. More definitive experiments that led to the same conclusion are discussed below.

Even though the mating results described above were unambiguous, other experiments were done to verify the counterclockwise orientation for both the *phoΩ87* and *phoΩ766* insertions. This involved conducting a series of P1 crosses with strains that had either a sense or antisense *lacZ* DNA insertion in the *phoA* gene nearby. Both the *phoA* and *phoB* genes are transcribed in a clockwise direction (27). The gene order is *phoA-proC-aroLM-phoBR-tsx*; the *phoA-phoBR* region is nonessential and can be deleted (31). In crosses between mutants with *lacZ* transposon insertions in the *phoA-phoBR* region, recombination can occur between homologous Mu d1 and λpl(209) sequences of a *phoA* and *phoBR* insertion that are in the same relative orientation. A recombination event between two such insertion elements leads to a deletion of the intervening sequences, which causes a Pro$^-$ phenotype. To test for the orientation of the *lacZ* DNA in this manner, *phoΩ87* and *phoΩ766* mutants were made with the *tsx-234*::Tn*10* insertion nearby, thus allowing for the selection of the Tn*10*-encoded tetracycline resistance marker in subsequent crosses. The recipients had either a Mu d1 mutation or a λp1(209)-stabilized version of it in the *phoA* gene; donors always had the λp1(209) version of the *phoBR* operon insertions, to avoid transposition during the crosses. Both the *phoΩ87* and *phoΩ766* insertions gave a high proportion (over 30%) of proC$^-$ recombinants, which also had other phenotypes that are expected for Δ(*phoA proC aroLM phoBR*)::Mu d1 or λp1(209) recombinants, when crossed with the *phoAΩ340* antisense Mu d1 or λp1(209) mutant. No recombinants of this type were found in crosses with the *phoAΩ4* sense Mu dl or λp1(209) mutant (C. Schmellik-Sandage and B. L. Wanner, unpublished data). These results provide independent evidence that both the *phoΩ87* and *phoΩ766* insertions are in the counterclockwise orientation, i.e., both are in an antisense orientation with respect to the *phoB* gene.

DISCUSSION

Two *phoΩ*Mu d1 mutants, which have simple insertions, simultaneously abolished *phoB* and *phoR* gene function in *cis*. One mutation mapped in the 5′ end of the *phoB* gene, and the other mapped upstream of the *phoB* structural gene, very likely within or near the *phoBR* operon promoter. The placement of this insertion upstream of the structural gene and near the promoter is consistent with the apparent leakiness (blue color) of the *phoΩ766* mutant on XP indicator agar and its ability to yield secondary mutants in which the

function of the *phoBR* operon is only partially restored. These results demonstrate an operon structure for the *phoB* and *phoR* genes in which *phoBR* gene function is expressed solely from a promoter(s) upstream of the *phoB* structural gene.

Two other laboratories reported results relevant to a possible *phoBR* operon structure. In one study, several *phoBΩTn5* mutations were identified that fail to show a polar effect on *phoR* gene function, thus leading Tommassen et al. (21) to conclude that the *phoB* and *phoR* genes are not in an operon. They used a high-copy plasmid carrying the *phoB* and *phoR* genes, but we obtained similar results with a chromosomal *phoBΩTn5* mutation (unpublished data). The results here show that chromosomal Mu dl mutations within or upstream of the *phoB* gene simultaneously abolished *phoB* and *phoR* gene function. Since regulatory proteins tend to be required in small amounts, the Tn*5* mutations apparently did not totally abolish downstream transcription. The lack of a polar effect with *phoΩTn5* mutations could be due to transcription of the *phoR* gene from a Tn*5* sequence or a failure of the Tn*5* elements to totally block transcriptional readthrough.

Makino et al. (15) examined the expression of a *phoR-lacZ* transcriptional fusion that was made on a plasmid in vitro. While they found that an increase in *phoR* transcription during phosphate limitation requires the upstream *phoB* gene promoter, their results did not show that the *phoR* gene lacks its own promoter. Indeed, expression of the *phoR-lacZ* transcriptional fusion is only partially dependent on an intact *phoB* promoter on the plasmid. Also, the *phoB* promoter is not required for *phoR* gene function when analogous *phoR*$^+$ plasmids are made lacking the *phoB* promoter, even though a low-copy-number plasmid is used (15). While their results showed that the *phoR* gene can be expressed from the *phoB* promoter during phosphate limitation, the possibility remained that a different promoter is responsible for the basal level of *phoR* expression, which is sufficient to maintain repression of the phosphate regulon when phosphate is in excess. The results here show that *phoR* gene function from the *phoBR* operon on the chromosome was solely dependent on expression from a site upstream of the *phoB* gene.

Expression of the *phoBR* operon is increased during phosphate limitation (9, 15, 19). This suggests that the *phoB* and *phoR* gene products are required in larger amounts to express fully the *phoBR*-dependent *psi* promoters that are coregulated. Smaller amounts may be required to maintain repression. It is also possible that an increased amount of the PhoR protein is required to reestablish the repressed state following a period of phosphate limitation. In any case, a *phoBR* operon structure has implications in the molecular regulation of the phosphate regulon *psi* genes that are involved in phosphorus assimilation (27).

It was interesting that one *phoΩ*Mu dl mutant expressed the *lacZ* reporter gene, albeit at a low level, and the other did not. The explanation that one *lacZ* reporter gene was in the sense orientation and the other was not failed because both Mu dl insertions proved to be oriented counterclockwise on the chromosome, i.e., in an orientation opposite to that of the *phoBR* operon. The small amount of *lacZ* expression in the *phoΩ87*::Mu dl mutant was also unaffected by phosphate or by a *phoB*$^+$ or *phoR*$^+$ gene in *trans* (Table 1). This result also differs from the expected characteristics for the *phoB* promoter itself, which is both phosphate regulated (9, 19) and autogenously controlled (9). It is perhaps relevant that other investigators (19) showed that two promoters exist within the vicinity of the *phoB* gene: the *phoB* gene promoter and another that expresses the *lacZ* reporter gene in the opposite orientation. They also showed that the other promoter is neither phosphate regulated nor autogenously controlled by the *phoB* gene product. Perhaps, their other promoter maps within the *phoB* gene and is responsible for *lacZ* expression in the *phoBΩ87* mutant. Since the *phoΩ87* mutation maps within or very near the 5′ end of the *phoB* structural gene, transcription may be due to the expression of an antisense promoter. Apparently this transcript is also terminated within or near the 5′ end of the *phoB* structural gene, because the upstream *phoΩ766* insertion is Lac$^-$. These results are consistent with a control of the *phoBR* operon by antisense RNA (17).

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