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# Mutational Analysis of an *Escherichia coli* Fourteen-Gene Operon for Phosphonate Degradation, Using Tn*phoA'* Elements

WILLIAM W. METCALF and BARRY L. WANNER*
*Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907*

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**All genes for phosphonate (Pn) utilization in *Escherichia coli* are in a large cluster of 14 genes named, in alphabetical order, *phnC* to *phnP*. Plasmids carrying these genes were mutagenized by using Tn*phoA'-1*, and 43 mutants containing simple insertions were studied in detail. Their insertion sites were defined by restriction mapping and by DNA sequencing. One or more mutations in each *phn* gene was identified. In 23 mutants, expression of the Tn*phoA'-1 lacZ* gene was phosphate starvation inducible. These mutants had Tn*phoA'-1* oriented in line behind the *phnC* promoter, i.e., in the + orientation. In 20 mutants, the Tn*phoA'-1 lacZ* gene was expressed at a low basal level. These mutants had insertions in the opposite orientation. All 43 *phn*::Tn*phoA'-1* insertions were recombined onto the chromosome to test for mutational effects, and their structures on the chromosome were verified by DNA hybridization. Those in the + orientation were switched to Tn*phoA'-9*, which has an outward promoter for expression of downstream genes. These insertions were tested for polar effects by measuring β-glucuronidase synthesis from a *uidA* gene transcriptionally fused to the 3' end of the *phnP* gene. The results indicate the following: (i) the *phnC*-to-*phnP* gene cluster is an operon of 14 genes, and the *phnC* promoter is the sole *psi* promoter; (ii) three gene products (PhnC, PhnD, and PhnE) probably constitute a binding protein-dependent Pn transporter; (iii) seven gene products (PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, and PhnM) are required for catalysis and are likely to constitute a membrane-associated carbon-phosphorus (C-P) lyase; (iv) two gene products (PhnN and PhnP) are not absolutely required and may therefore be accessory proteins for the C-P lyase; and (v) two gene products (PhnF and PhnO) are not required for Pn use and may have a regulatory role because they have sequence similarities to regulatory proteins. The mechanism for breaking the C-P bond by a lyase is discussed in light of these results.**

Phosphorus (P) acquisition and assimilation are of fundamental importance in cell physiology because P is a required nutrient. To fulfill this requirement, *Escherichia coli* has evolved several gene systems whose products allow for P assimilation from a variety of compounds (26–28). These compounds include ones for high-affinity $P_i$ transport, for uptake of glycerol-3-phosphate, for hydrolysis of phosphomonoesters, for passage of polyanions into the periplasm, and for the degradation of phosphonates (Pn), which is the subject of this paper.

Pn are similar to phosphate esters but have a direct carbon-phosphorus (C-P) bond instead of a carbon-oxygen-phosphorus (C-O-P) bond. Unlike the C-O-P bond, the C-P bond can be extremely stable. In some cases, the C-P bond has a bond energy similar to that of a C-C bond. Two degradative pathways, the phosphonatase and C-P lyase pathways, are probably for Pn use as a P source because the genes for these pathways are under PHO regulon control. A third pathway for Pn breakdown may be involved in their use as a carbon source because it is not under PHO regulon control (reviewed by Wanner and Metcalf [30]). The distribution of Pn degradative pathways among enteric bacteria varies. In *E. coli*, Pn are broken down solely by the C-P lyase pathway; in *Enterobacter aerogenes*, Pn are broken down by both the C-P lyase and phosphonatase pathways; and in *Salmonella typhimurium*, Pn are broken down only by the phosphonatase pathway. The corresponding genes for each of these pathways are regulated much like other members of the PHO regulon (11, 14, 29).

The biochemical characterization of the C-P lyase pathway has proven to be quite difficult. Despite numerous attempts, the detection of C-P lyase activity in a cell-free system has been unsuccessful. In most cases, this activity has been lost following cell lysis (8, 23). Although one laboratory (21) had reported an in vitro activity, that report was later shown to be incorrect. In that instance, the activity had been determined by measurement of $P_i$ release in an assay containing protein components from an *Enterobacter aerogenes* cell extract. It was later shown that $P_i$ was released from such extracts in the absence of an added substrate (13).

As a means to study the C-P lyase without a biochemical assay, genetic and molecular studies on the genes for this pathway were undertaken (7, 17, 24, 29). All known genes for Pn degradation in *E. coli* are in a large cluster of 14 genes, the *phnCDEFGHIJKLMNOP* gene cluster, that appear to be transcribed from a single promoter upstream of the *phnC* gene (Fig. 1). On the basis of genetic and molecular evidence, roles have been suggested for seven *phn* gene products. Accordingly, the PhnD and PhnE proteins may have a role in Pn uptake; the PhnH, PhnJ, PhnK, and PhnP proteins may be components of the C-P lyase; and the PhnO protein may have a role in gene regulation. These conclusions are based on results from studying the effects of transposon-induced mutations in these *phn* genes on the use of various P-containing compounds (17, 19). They relied on the interpretation that insertion elements carrying an outward promoter for downstream gene expression would frequently

* Corresponding author.

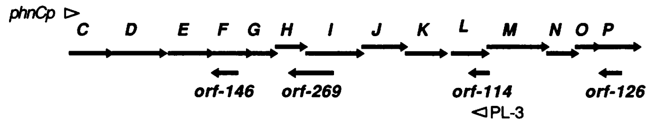


FIG. 1. Organization of the *phn* gene cluster. The rightward arrows show 14 genes, named *phnC* to *phnP*, that were shown to constitute the minimum region required for Pn use (19; this paper). These genes appear to be transcribed from a single $P_i$-regulated promoter immediately upstream of the *phnC* gene. Four ORFs with appropriately spaced consensus ribosome binding sites and a consensus promoter, called PL-3, are shown on the opposite strand (7).

cause nonpolar mutations. Effects due to polarity could not be unambiguously ruled out, however. Furthermore, it is not known which of the seven other *phn* genes have roles in Pn metabolism.

In this paper, further mutational studies on the *phn* gene cluster are reported. Our earlier mutational strategy was modified to aid in the determination of roles for all 14 *phn* genes. In particular, a promoterless *uidA* cassette that encodes β-glucuronidase (20) was introduced immediately downstream of the *phnP* gene (19). This allowed determination of whether mutational effects were caused by the transposon insertions or polarity. In addition, new $Pn^+$ plasmids designed for use in transposon mutagenesis and allele replacement were constructed (19).

The modified strategy involved mutagenesis of these $Pn^+$ plasmids by using a Tn*phoA'* element (31). The *phn*::Tn*phoA'* insertions were then recombined onto the chromosome to test for mutational effects due to gene disruption or to transcriptional polarity. These effects were distinguished by measuring β-glucuronidase synthesis in mutants containing the $PhnP^+$ *phnP-uidA* fusion. Appropriate Tn*phoA'* insertions were also switched to Tn*phoA* and other Tn*phoA'* elements to allow testing for transcriptional, translational, and cell surface localization determinants at different sites in the *phn* gene cluster. The results from the mutational studies are described in this paper. Studies on the expression of these and other *phn*::Tn*phoA* and *phn*::Tn*phoA'* fusions are in progress (15).

## MATERIALS AND METHODS

**Media, chemicals, and enzymes.** In general, media, chemicals, and enzymes were the same as previously reported (29). Tryptone-yeast extract (TYE) agar with an appropriate antibiotic(s) was used for selection of transformants, transductants, and exconjugants. Ampicillin was used at 100 μg/ml for plasmid-borne resistance and at 25 μg/ml for chromosomal resistance. Kanamycin was used at 50 μg/ml. Streptomycin was used at 200 μg/ml for ribosomal (*rpsL*) resistance. Streptomycin and spectinomycin were used in combination at 35 μg/ml each for resistance encoded by the *aadA* gene. Tetracycline was used at 15 μg/ml with 2.5 mM sodium $PP_i$ for resistance due to chromosomal *tetAR* genes and at 6 μg/ml for resistance due to plasmid *tetAR* genes. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XG; Bachem, Torrance, Calif.) was used at 40 μg/ml for the detection of β-galactosidase. Isopropyl-β-D-thiogalactoside (IPTG) was added at $3.3 \times 10^{-4}$ M for induction of *lacP*-promoted *lacZα* expression in $lacI^{q1}$ hosts. Tetracycline-sensitive ($Tc^s$) recombinants were selected on tetracycline-sensitive-selective (TSS) agar prepared as described elsewhere (6). Restriction endonucleases, enzymes for DNA sequencing, T4 DNA ligase, and T4 DNA polymerase (New England Biolabs, Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; Promega, Madison, Wis.; or U.S. Biochemical Corp., Cleveland, Ohio) were used according to the manufacturers' specifications.

**Tests for P assimilation phenotypes.** P compounds were prepared at 0.1 M, filter sterilized, and added to glucose 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium to test for use. Cells were tested by streaking onto glucose MOPS agar containing each compound at 0.1 mM as the sole P source. Appropriate positive and negative control strains were always compared side by side on the same plate. This was necessary to control for the small (and sometimes variable) amount of growth seen for all strains because of contaminating P compounds in various medium components. The phosphate-starvation-inducible (Psi)–$LacZ^+$ phenotype was scored by comparison of colony color on glucose MOPS agar containing XG and 2.0 or 0.1 mM $P_i$. Plasmid strains were tested for the Psi-$LacZ^+$ phenotype on agar containing ampicillin.

**Bacteria.** Bacteria are described in Table 1. BW15818 was used as a host for plasmids requiring the Π protein, the *pir* gene product, for DNA replication. λ$pir^+$ lysogens of S17-1, BW17068 and BW17272, were used for transposon mutagenesis. BW17068, like S17-1, carries the *supE44* amber suppressor allele, whereas BW17272 does not. BW14877 was used as a host for pKUN19 clones. Mutational studies were done with strains or plasmids carrying the $Pn^+$ *phn*(EcoB) locus from *E. coli* B (29). The cryptic *E. coli* K-12 locus is denoted *phn*($EcoK^0$). The Δ(*mel phnP*)*2* deletion has one end within the *melRAB* locus more than 20 kbp upstream of the *phnC* gene and the other within the *phnP* gene. The Δ(*phn*)*33-30* deletion has one end near the 3' end of the *phnC* gene and the other more than 20 kbp downstream of the *phnP* gene (17, 29). Strains described previously included λ$pir^+$ lysogens of S17-1 (20) and SM 10 (31).

**Plasmids and phages.** The $Pn^+$ plasmids pBW120, pWM1, pWM9, and pWM10 are shown in Fig. 2. The plasmids pDK185, pDK184, and pDK183 (1) contain the 1.2-kbp $EcoRI_1$-to-$EcoRV_2$, the 2.8-kbp $EcoRI_1$-to-$EcoRV_3$, and the 4.2-kbp $EcoRI_1$-to-$EcoRV_4$ fragments (Fig. 3), respectively, cloned before the *lacZ* gene of pMB1 replicon pRS415 (22). The phages λRZ5(DK185), λRZ5(DK184), and λRZ5(DK183) contain the *phnCD'-lacZ*, *phnCDE-lacZ*, and *phnCDEFGH'-lacZ* fusions, respectively. These phages were made by crossing the fusions from the respective plasmid onto λRZ5 by homologous recombination (10). To do this, a λRZ5 lysogen of BW9766 was transformed with these plasmids, the resultant transformants were UV irradiated to make mixed lysates, the lysates were used to infect BW14893, and ampicillin-resistant ($Amp^r$) transductants were selected on TYE agar containing ampicillin at 25 μg/ml, to select for lysogens carrying λRZ5 recombinant phage. These lysogens were UV irradiated, and the resultant phages were plaque purified to prepare new lysates, as described elsewhere (2). Strains were subsequently lysogenized with these phages by plating cells with phage in top agar, and $Amp^r$ lysogens were isolated by streaking plaque centers onto TYE agar containing ampicillin at 25 μg/ml. Phages and plasmids described previously included M13mp18 (16), λG217 (31), λRZ5 (2), λ::Tn*5-112* (17), λ::Tn*phoA'-1* (31), λ::Tn*phoA-132* (31), pCS3 (19), pKUN19 (16), and pMW11 (31).

**Isolation of *phn*::Tn*phoA'-1* mutants.** The λ$pir^+$ S17-1 derivatives BW17068(pWM9), BW17068(pWM10), and BW 17272(pWM9) were infected with λ*cI857 b221 Pam3 rex*::Tn*phoA'-1* (also called λ::Tn*phoA'-1*), and kanamycin-resistant ($Kan^r$) transductants were selected. Plates contain-

TABLE 1. Bacterial strains

| Strain[a] | Genotype | Pedigree | Source, construction, or reference |
|---|---|---|---|
| BW3908 | Δ*lac-169 phn*(EcoK⁰) *creB510 rpsL267 thi* | XPh1a via BW1589 (25) | Aro⁺ with P1 on BD792 (25) |
| BW5045 | Δ*lac-169 srlC300*::Tn*10* | BD792 via BW3414 (25) | Tc^r with P1 on MC4100 *srlC300*::Tn*10 recA1* |
| BW9766 | Δ*lac-169 creB510 thi* Δ(*phnC?DEFGHIJKLMNOP*)*33-30* | XPh1a via BW2930 | 29 |
| BW7089 | Δ*lac-169 zbf-5*::Tn*10* Δ(*psiF proC aroLM phoBR*)*9-6 creB510 thi* | XPh1a via BW3212 (25) | Tc^r with P1 on P2217 |
| BW14003 | DE3(*lac*)X74 *mel*::Tn*5*seq1 *phnP3*::Tn*5-132* Δ*phoA532 arcA** | $MG1655_{MC}$ via BW13719 | 17 |
| BW14109 | DE3(*lac*)X74 Δ(*mel proP phnCDEFGHIJKLMNOP*)*3*::Tn*5* seq1/*132*(*kan*) Δ*phoA532 arcA** | $MG1655_{MC}$ via BW14003 | Tc^s on TSS agar[b] |
| BW14329 | Mu-1 Δ*lac-169* Δ(*mel proP phnCDEFGHIJKLMNOP*)*2*::Tn*5* seq1/*132*(*tet*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14295 | 11 |
| BW14877 | Δ(*mel proP phnCDEFGHIJKLMNOP*)*3*::Tn*5* seq1/132(*tet*) DE3(*lac*)X74 Δ*phoA532 arcA** *recA*::*cml-aadA* | $MG1655_{MC}$ via BW14108 | 19 |
| BW14879 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phn*(EcoB) *arcA** | $MG1655_{MC}$ via BW13688 | 16 |
| BW14893 | DE3(*lac*)X74 Δ*phoA532* Δ(*phnC?DEFGHIJKLMNOP*)*33-30* | BD792 via BW14331 | 11 |
| BW14902 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnE1*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13196 (17) |
| BW14903 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnP2*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13197 (17) |
| BW14904 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnP3*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13199 (17) |
| BW14905 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnH4*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13201 (17) |
| BW14906 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnO5*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13202 (17) |
| BW14907 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnK6*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13204 (17) |
| BW14908 | pMW11 Muc*62*(Ts⁻) DE3(*lac*)X74 Δ*phoA532 phnJ7*::Tn*5-112 arcA** | $MG1655_{MC}$ via BW14879 | Kan^r with P1 on BW13205 (17) |
| BW15818 | λ*pir*⁺ Δ*lac-169* Δ(*mel proP phnCDEFGHIJKLMNOP*)*2*::Tn*5* seq1/132(*tet*) *creB510 hsdR514 rpsL* | BD792 via BW14521 (19) | Tc^r with P1 on BW14001 (17) |
| BW16877 | DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532* Δ(*fumCA manA uidAR add*) | BD792 via BW16846 | 20 |
| BW17006 | λ*pir*⁺ Δ*lac-169* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 hsdR514 rpsL malT* | BD792 via BW16788 (19) | Spontaneous λvir^r; Mal⁻ |
| BW17068 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant; *recA proA creB510 hsdR17 endA1 supE44 thi* | S17-1 | 20 |

*Continued on following page*

TABLE 1.—*Continued*

| Strain[a] | Genotype | Pedigree | Source, construction, or reference |
|---|---|---|---|
| BW17090 | pWM9 λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *recA proA creB510 hsdR17 endA1 supE44 thi* | S17-1 via BW17068 | Amp$^r$ with pWM9 |
| BW17091 | pWM10 λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7; integrant *recA proA creB510 hsdR17 endA1 supE44 thi* | S17-1 via BW17068 | Amp$^r$ with pWM10 |
| BW17204 | DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532 rpsL* | BD792 via BW14332 | 20 |
| BW17208 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *srlC300*::Tn*10 proA creB510 hsdR17 endA1 supE44 thi* | S17-1 via BW17068 | Tc$^r$ with P1 on BW5045 |
| BW17209 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *srlC300 proA creB510 hsdR17 endA1 supE44 thi* | S17-1 via BW17208 | Tc$^s$ on TSS agar |
| BW17267 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *srlC300 creB510 hsdR17 endA1 supE44 thi* | S17-1 via BW17209 | Pro⁺ with P1 on BW13033 (31) |
| BW17268 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *srlC300 creB510 hsdR17 endA1 zbf-5*::Tn*10 thi* | S17-1 via BW17267 | Tc$^r$ with P1 on BW7089 |
| BW17272 | λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *recA*::*cml-aadA creB510 hsdR17 endA1 zbf-5*::Tn*10 thi* | S17-1 via BW17268 | Cm$^r$ with P1 on BW10724 (29) |
| BW17277 | pWM9 λ*pir*⁺ RP4-2-*tet*::Mu-1*kan*::Tn7 integrant; *srlC300 creB510 hsdR17 endA1 zbf-5*::Tn*10 thi* | S17-1 via BW17272 | Amp$^r$ with pWM9 |
| BW17369 | DE3(*lac*)X74 Δ*phoA532 phn*(EcoB) *phn-10*::*uidA2-aadA fumB** Δ(*fumCA manA uidAR add*) | BD792 via BW17366 (19) | Spc$^r$/Str$^r$ with P1 on BW16887 (20) |
| BW17399 | DE3(*lac*)X74 *recD1901*::Tn*10* Δ*phoA532 rpsL phn*(EcoB) | BD792 via BW17204 | 19 |
| BW19093 | DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532 uidA*(Δ*Mlu*I)::*pir*⁺ | BD792 via BW16877 | 18 |
| BW19100 | DE3(*lac*)X74 Δ*phoA532* Δ(*mel proP phnCDEFGHIJKLMN OP*)*3*::Tn*5*seq1/*132*(*kan*) *uidA* (Δ*Mlu*I)::*pir*⁺ | BD792 via BW19093 | Kan$^r$ with P1 on BW14109 |
| P2217 | *lacZ*(Am) *trp*(Am) *zbf-5*::Tn*10 supE44 rpsL thi* | | P. Reeves |

[a] All bacteria are *E. coli* K-12 derivatives.
[b] The Δ(*mel proP phnCDEFGHIJKLMNOP*)*3*::Tn*5*seq1/*132*(*kan*) mutation was formed by homologous recombination between IS*50* sequences in the *mel*::Tn*5*seq1 and *phnP3*::Tn*5-132* insertions, in accordance with procedures described previously (17).

ing about 5,000 Tn*phoA'-1* mutants were mated by replica plating onto a lawn of BW17006 (λ*pir*⁺ *rpsL malT*) on TYE agar. These plates were incubated at 37°C for about 3 h and then replica plated onto TYE agar containing streptomycin and kanamycin, to select exconjugants carrying pWM9::Tn*phoA'-1* or pWM10::Tn*phoA'-1* mutant plasmids. As expected, the yield of Kan$^r$ transductants was much greater with the suppressor-negative host BW17272 than with the *supE44* host BW17068. Also, the efficiency of mutagenesis was greatly enhanced by using the λ$^r$ *malT* mutant BW17006 as a recipient.

**Molecular genetics.** P1 transductions using P1*kc*, DNA transformation, M13 growth, and conjugations were carried out as previously described (25, 29). λG217 (*imm434 cI-2* Δ*int-9 h80*) was used to test for phage 434 immunity. The *phn*::Tn*5-112* insertions were cloned by using the mini-Mu cloning vector pMW11 (29). pMW11 is a pMB1 replicon carrying spectinomycin and streptomycin resistance. To clone *phn*::Tn*5-112* insertions by using pMW11, each insertion was crossed into BW14879 with P1 by selection for Kan$^r$ transductants (Table 1). Lysates of these strains were made by heat induction and used to infect the Mu-1 lysogen BW14329. In some cases, Kan$^r$ transductants were selected directly. In other cases, streptomycin- and spectinomycin-resistant (Str/Spc$^r$) were selected first and then Kan$^r$ transductants were selected by replica plating.

Recombinational switching of Tn*5-132*, Tn*phoA*, and Tn*phoA'* elements was done as described previously (17, 31). In brief, mutants carrying an insertion due to one element were infected with a λ*P*am phage carrying an

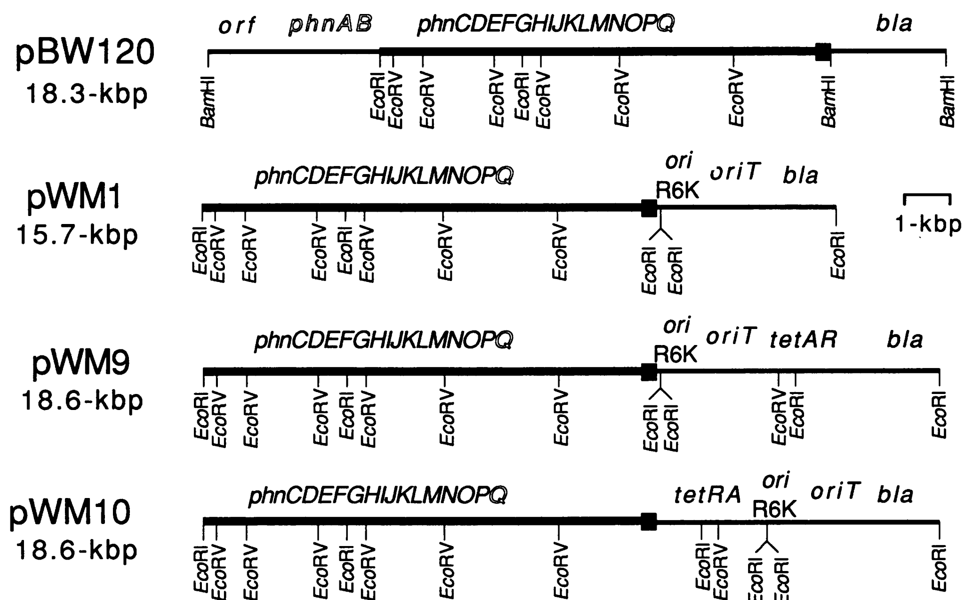


FIG. 2. Structures of Pn$^+$ plasmids. pBW120 (29) has a 15.6-kbp *Bam*HI fragment, for which the sequence has been determined (7), cloned into pUC18. pWM1, pWM9, and pWM10 contain the 11.4-kbp *Eco*RI-to-*Bam*HI fragment from pBW120 cloned into a vector having the replication origin, *ori*, from R6K and the transfer origin, *oriT*, from RP4 (19). Hollow letters indicate the *phnA*, *phnB*, and *phnQ* ORFs that were shown not be required for Pn use. pWM9 and pWM10 contain two *Nsi*I sites within the *tetAR* genes; pWM1, pWM9, and pWM10 contain *Bam*HI sites in vector sequences (not shown).

element with a different drug resistance. Transductants selected for resistance to the new antibiotic resistance marker were tested for ones that lost the parental resistance marker while retaining the appropriate mutant phenotype.

**Allele replacement.** The *phn*::Tn*phoA'-1* insertions were recombined onto the chromosome by either of two methods. One method involved conjugative transfer of *pir*-dependent plasmids into a Rec$^+$, non-*pir* host, in which such plasmids are maintained by integration into homologous chromosomal sequences. Since pWM9 and pWM10 carry the *tetAR* genes, segregants that subsequently lost vector sequences were selectable as Tc$^s$ ones. Many pWM9::Tn*phoA'-1* and pWM10::Tn*phoA'-1* insertions were recombined onto the chromosome in this way by conjugative transfer into BW17204. Accordingly, Kan$^r$ and Str$^r$ exconjugants were selected and purified once nonselectively. Then Tc$^s$ derivatives were selected, purified once nonselectively, and tested for ones that retained the *phn*::Tn*phoA'* insertion. When this method was used, it was important to show that the suspected integrants had not simply become lysogenic for λ*pir*$^+$, because the donor releases this phage. This was done by testing for sensitivity to λG217 (*imm434 cI*$^-$) because λ*pir*$^+$ carries the phage 434 immunity region. Alternatively, *phn*::Tn*phoA'-1* insertions were recombined onto the chromosome by transformation of BW17399 [*recD phn*(EcoB)] with linear plasmid DNAs.

**Molecular biology techniques.** Plasmid DNA isolation and recombinant DNA methods were in general the same as described previously (2). Chromosomal and single-stranded phage DNAs were isolated as described elsewhere (4). Gel-purified DNA fragments were excised from low-melting-point agarose gels prepared with Tris-borate buffer, and purified by using Elutip-D columns (Schleicher & Schuell, Inc., Keene, N.H.) according to the manufacturer's recommendations. Chromosomal DNAs from the *phn*::Tn*phoA'-1* mutants were analyzed by DNA hybridization using randomly primed, [α-$^{32}$P]dATP-labeled, *Bam*HI-digested pBW120 as a probe, as described previously (29).

Sequencing of double-stranded DNA templates was done as previously described (18). Primer a (5'GTCACATGGA AGTCAGAT3') was used for determination of Tn*5-112* junctions (Fig. 4). Primer b (5'AATATCGCCCTGAGCA3') was used for determination of the junction at the *phoA* end of Tn*phoA'* elements. Subclones suitable for sequencing of chromosomal-Tn*5-112* junctions were generated by digestion of the mini-Mu clones with *Sma*I and by ligation of the

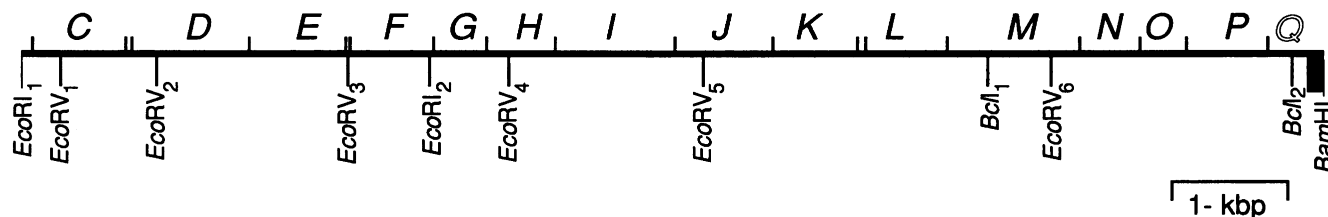


FIG. 3. Restriction map of the *phnCDEFGHIJKLMNOP* gene cluster. The *phn* genes in pWM9 and pWM10 are shown. All sites for enzymes indicated are shown and correspond to chromosomal DNA, except that the *Bam*HI site was from a cloning vector. The rightmost 114 bp are Mu DNA (19, 29).

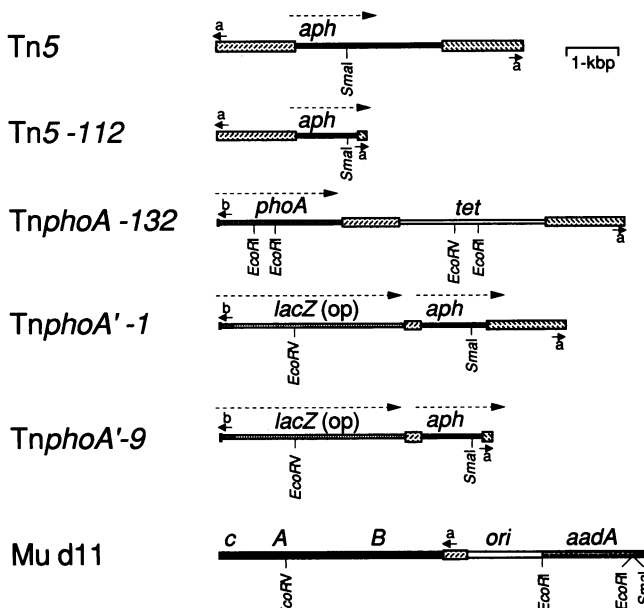


FIG. 4. Physical structures of Tn*5*, Tn*5-112*, Tn*phoA-132*, Tn*phoA'-1*, Tn*phoA'-9*, and Mu d11. Like Tn*5*, Tn*phoA-132* and Tn*phoA'-1* are transposition proficient. Tn*5-112* and Tn*phoA'-9* are transposition defective (17, 31). The *aph* gene encodes an aminoglycoside phosphotransferase for kanamycin or neomycin resistance. The *tet* genes in Tn*phoA-132* are from Tn*10* and encode tetracycline resistance. The *aadA* gene in Mu d11 encodes aminoglycoside adenyltransferase for streptomycin and spectinomycin resistance. Tn*phoA-132*, like Tn*phoA*, can form *phoA* gene fusions. Tn*phoA'-1* and Tn*phoA'-9* can form *lacZ* transcriptional, i.e., operon (op), fusions. Dashed arrows show the direction of transcription for the *aph*, *phoA*, and *lacZ* (op) genes. Transcription from the *aph* promoter proceeds outward into adjacent sequences for Tn*5-112* and Tn*phoA'-9*. Hatched boxes represent IS*50* DNA. Arrows marked a and b show the locations of IS*50*- and *phoA*-specific primers, respectively. Mu d11 is the mini-Mu cloning element in pMW11 (31). Like similar mini-Mu elements, Mu d11 has 4,364 bp of the Mu left end joined to a segment of IS*50* in the Mu *cim* gene, as determined by DNA sequencing using primer a. All sites for enzymes indicated are shown.

chromosomal-IS*50*L-*aph* fragment to *Sma*I-digested pKUN19. Amp$^r$ and Kan$^r$ transformants of BW14877 were selected. Primers a and b were synthesized at the Laboratory for Macromolecular Structure at Purdue University.

**Enzyme assays.** β-Glucuronidase assays were performed as previously described (20), using cell extracts from cultures grown overnight in 0.4% glucose MOPS liquid medium with 0.2 mM $P_i$. Cultures with these concentrations of glucose and $P_i$ are $P_i$ limited (data not shown). Extracts were made by collecting cells from 10-ml cultures by centrifugation and by resuspension in 0.5 ml of assay buffer (50 mM sodium phosphate [pH 7.0], 10 mM 2-mercaptoethanol, 100 μg of chloramphenicol per ml). The cells were sonicated on ice three times for 30 s each with a model W185 Sonifier Cell Disrupter (Heat Systems-Ultrasonics Inc., Plainview, N.Y.) at 35 W. Cell debris was removed by centrifugation, and protein was determined by the method of Lowry et al. (11a) with bovine serum albumin as the standard. Units are nanomoles of *p*-nitrophenol produced per minute. Specific activity is in units per milligram of protein.

## RESULTS

**Construction of *phnQ* insertions.** Two experiments were done to determine whether the *phnQ* open reading frame (ORF) was required for Pn use. First, a *lacZ* cassette was inserted as a *Bam*HI-to-*Bgl*II fragment into the *Bcl*I site of the *phnQ* sequence (Fig. 3). This was done by partial digestion of pWM1 with *Bcl*I and by ligation to a *Bam*HI-to-*Bgl*II *lacZ* cassette from pCS3 (20). Two kinds of pWM1 recombinant plasmids were characterized. One kind had the *lacZ* cassette substituted for the *Bcl*I$_1$-to-*Bcl*I$_2$ fragment; the other had the *lacZ* cassette in the *phnQ Bcl*I site. These plasmids were tested for complementation of the Δ*phn* mutant BW19100, which has a deletion of the 3' end of the *phn* gene cluster. Plasmids with the *phnMNOPQ* interval deleted were Pn$^-$, as expected. In contrast, *phnQ*::*lacZ* plasmids were Pn$^+$. Therefore, the *phnQ* ORF is not required for Pn use, and the *phnP* gene is the last gene in the *phn* gene cluster. On the basis of these results, a *Sac*I site was introduced by site-directed mutagenesis immediately after the tandem stop codons of the *phnP* gene, which is within the 5' end of the *phnQ* coding region. Mutants having a *uidA* cassette in this *Sac*I site were also Pn$^+$ (19).

**Determination of *phn*::Tn*5-132* insertion sites.** The approximate locations of seven *phn*::Tn*5-132* insertions (*phnE1*::Tn*5-132*, *phnH4*::Tn*5-132*, *phnJ7*::Tn*5-132*, *phnK6*::Tn*5-132*, *phnO5*::Tn*5-132*, *phnP2*::Tn*5-132*, and *phnP3*::Tn*5-132*) had been previously determined by deletion mapping in P1 crosses (17). To define these sites more precisely, each insertion was switched to the transposition-defective Tn*5-112* element to eliminate problems due to transposition. These *phn*::Tn*5-112* insertions were cloned by using a mini-Mu cloning vector as described in Materials and Methods. The chromosomal-IS*50*L-*aph* fragments were then subcloned into pKUN19 as *Sma*I fragments in order to separate IS*50*L and IS*50*R sequences, and the junctions were determined by DNA sequencing using primer a (Fig. 4). The sites of all seven insertions corresponded closely to locations predicted earlier (Fig. 5).

**Isolation and characterization of *phn*::Tn*phoA'-1* mutants.** The transposon Tn*phoA'-1* was used to isolate new *phn* mutants. This transposon can form *lacZ* transcriptional fusions and can be switched to Tn*phoA* and other Tn*phoA'* elements (31). To do this, transfer-proficient transformants carrying the Pn$^+$ plasmid pWM9 or pWM10 were infected with λ::Tn*phoA'-1*, and Kan$^r$ transductants were selected. The transductants were then replica mated with the λ-resistant, λ*pir*$^+$ host BW17006 (*rpsL* Δ*phn*), and Str$^r$ and Kan$^r$ exconjugants were selected as described in Materials and Methods. A total of 643 exconjugants carrying pWM9::Tn*phoA'-1* or pWM10::Tn*phoA'-1* mutant plasmids were characterized.

All exconjugants were tested for Pn use and for a Psi-LacZ$^+$ phenotype. They were also tested for ampicillin and tetracycline resistance, to eliminate those plasmids with deletions or rearrangements. Altogether, 83 of the 643 exconjugants were Pn$^-$ or Psi-LacZ$^+$, or both. Plasmid DNAs were purified from these 83 mutants and from 20 randomly chosen Pn$^+$ exconjugants, and these DNAs were examined by digestion with *Eco*RI and *Eco*RV. pWM9 and pWM10 contain four *Eco*RI and seven *Eco*RV fragments greater than 100 bp in length (Fig. 2). Since Tn*phoA'-1* has one *Eco*RV and no *Eco*RI site (Fig. 4), examination of the *Eco*RI and *Eco*RV restriction patterns gave a fairly accurate approximation of the site for each insertion. As expected, none of the 20 randomly picked Pn$^+$ mutants had insertions in the

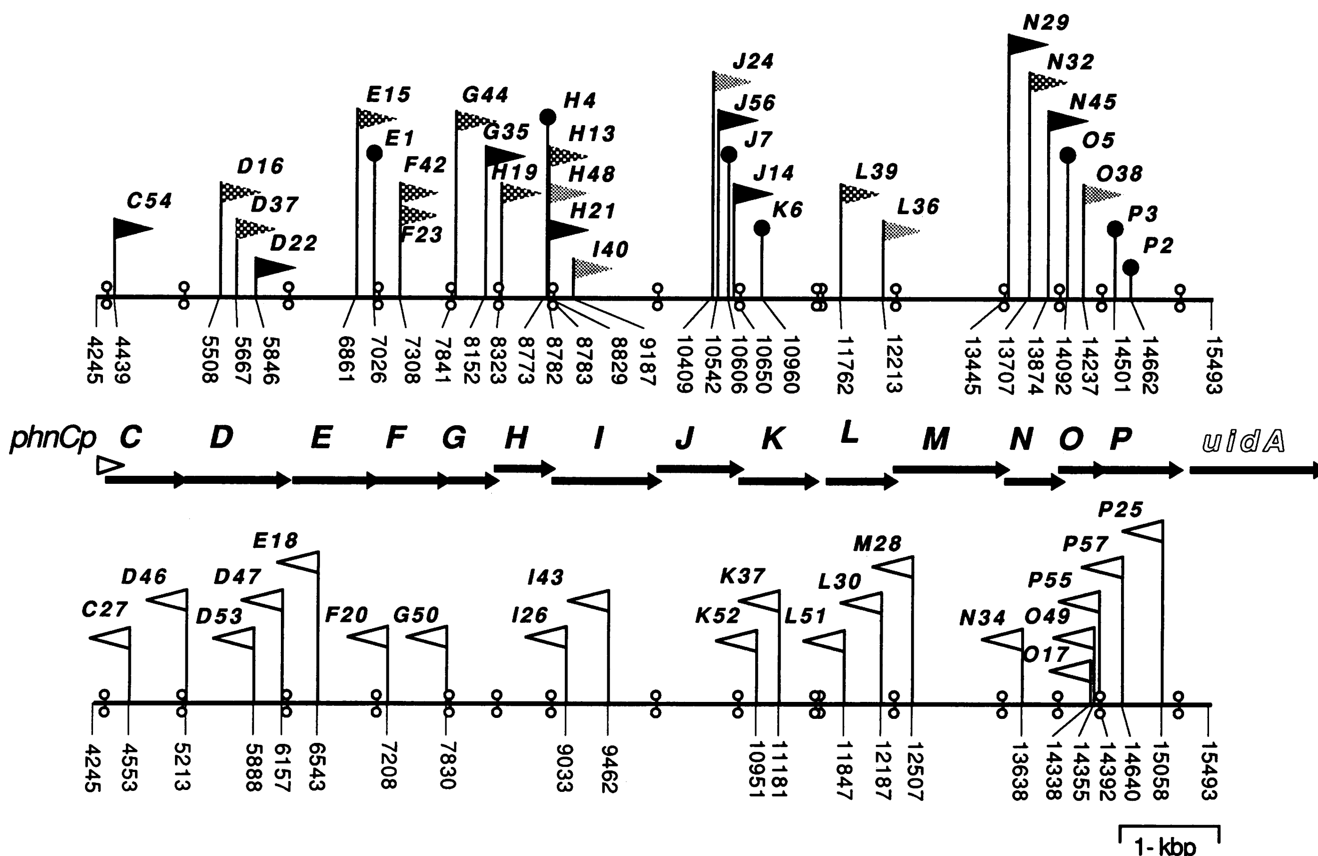


FIG. 5. Sites of *phn*::Tn*5-112* and *phn*::Tn*phoA'-1* insertions. Chromosomal DNA in pWM9 and pWM10 extends from an *Eco*RI site at position 4245 to base 15,493, as shown on the top and bottom lines. The top line shows all Tn*5-112* insertions and those Tn*phoA'-1* insertions in the + orientation. The bottom line shows those Tn*phoA'-1* insertions in the − orientation. Filled circles indicate sites of Tn*5-112* insertions, and flags indicate sites of Tn*phoA'-1* insertions. The shading of the flags indicates the reading frame at the insertion site. Solid flags indicate insertions after the first base of the interrupted codon. These would form in-frame gene fusions when switched to Tn*phoA* and Tn*phoA'* elements that can form gene fusions. Checked flags indicate insertions after the second base, and gray flags indicate insertions after the third base. Dumbbells mark the starts and stops of the *phn* genes, which are shown in the middle as solid arrows. Numbering is according to Chen et al. (7). The first upstream base is given for Tn*5-112* insertions and Tn*phoA'-1* insertions in the + orientation. The first downstream base is given for Tn*phoA'-1* insertions in the − orientation.

*phn* region. Most of these had simple insertions in the plasmid backbone.

Of the 83 $Pn^-$ or Psi-$LacZ^+$ pWM9::Tn*phoA'-1* and pWM10::Tn*phoA'-1* mutant plasmids, only 43 had simple insertions, on the basis of restriction mapping. The remainder had small deletions or additional insertions elsewhere. Of the 43 with simple insertions, 42 were originally identified because they showed an altered Pn phenotype. One $Pn^+$ mutant was characterized because it showed a Psi-$LacZ^+$ phenotype. Of the 42 $Pn^-$ mutants, 22 also displayed a Psi-$LacZ^+$ phenotype. The sole $Pn^+$ Psi-$LacZ^+$ insertion, the *phnN45* allele, was later shown to cause a weak $Pn^+$ phenotype when tested on the chromosome (see below).

**DNA sequence analysis of *phn*::Tn*phoA'-1* junctions.** The sites of the 43 $Pn^-$ or Psi-$LacZ^+$ *phn*::Tn*phoA'-1* insertions were determined by DNA sequencing using primer b, which is specific for the *phoA* end of Tn*phoA'-1* (Fig. 4). All 23 insertions that led to a Psi-$LacZ^+$ phenotype had Tn*phoA'-1* oriented in line behind the *phnC* promoter, hereafter denoted as the + orientation. The other 20 insertions that caused a $Pn^-$ phenotype had Tn*phoA'-1* in the opposite orientation, denoted as the − orientation (Fig. 5). In all cases, the sites determined by DNA sequencing were consistent with the approximate locations determined by restriction mapping. At least one Tn*phoA'-1* insertion was located within each of the 14 *phn* genes. Furthermore, the sites were nearly equally represented among the three possible reading frames. Somewhat surprisingly, two independent mutations had Tn*phoA'-1* insertions at the same site in the *phnF* gene.

**Allele replacement of *phn*::Tn*phoA'-1* insertions.** All 43 *phn*::Tn*phoA'-1* insertions were recombined onto the chromosome. Roughly two-thirds were recombined onto the chromosome by conjugative transfer into BW17204 and by selection for $Tc^s$ recombinants. This method was quite efficient for Tn*phoA'-1* insertions near the middle of the *phn* insert of pWM9 or pWM10, i.e., within the *phnEFGHI JKLM* region. For these insertions, 15 to 50% of the $Tc^s$ recombinants retained the *phn*::Tn*phoA'-1* insertion on the chromosome. However, this method was inefficient for insertions near either end of the insert. For these, only a small fraction of the $Tc^s$ segregants retained the *phn*:: Tn*phoA'-1* insertion. In the case of the *phnD16*::Tn*phoA'-1* allele, only 3 of 300 $Tc^s$ recombinants retained Tn*phoA'-1*. Also, in some cases, very large numbers of recombinants

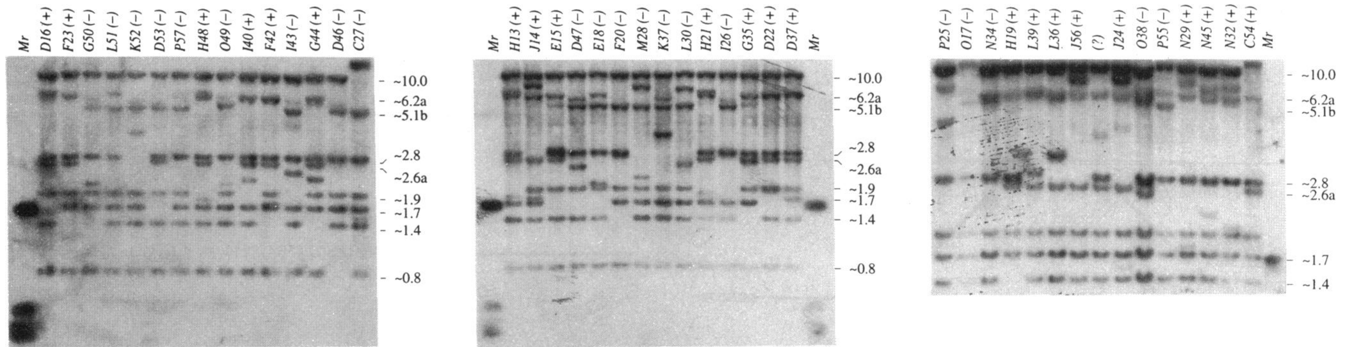


FIG. 6. DNA hybridization of chromosomal *phn*::Tn*phoA'-1* insertions. DNAs from each mutant were digested with *Eco*RV, separated by agarose gel electrophoresis, blotted, and examined by hybridization with pBW120 as the probe. The + and − signs indicate the orientation of each *phn*::Tn*phoA'-1* insertion. Chromosomal fragments that would hybridize for a wild-type *phn*(EcoB) strain are 10, 2.8, 1.7, 1.9, 1.4, and 0.8 kbp in length, along with a fragment(s) for the 3′ end. Since DNAs from + orientation mutants were isolated from transductants with the downstream *phn-10*::*uidA-aadA* insertion, two additional fragments of 6.2 and 2.6 kbp in length for the 3′ end, indicated as 6.2a and 2.6a, respectively, would also hybridize for these mutants. The 2.6-kbp band corresponds to *phn* DNA. Since DNAs from − orientation mutants were isolated from transductants that lost the *phn-10*::*uidA-aadA* insertion, a single additional fragment of 5.1 kbp in length for the 3′ end, indicated as 5.1b, would hybridize for these mutants. Since Tn*phoA'-1* has an internal *Eco*RV site, each mutant was expected to lose one particular fragment and to gain two new ones of predictable sizes. Also, three bands are expected to hybridize in lanes marked *Mr* because they correspond to fragments in the 1-kbp ladder DNA markers (Gibco-BRL, Gaithersburg, Md.) which are homologous to vector sequences in pBW120. With one exception, all DNAs gave the expected pattern. The exceptional case is marked with a question mark; it was for a recombinant that also showed an unexpected Pn phenotype (see text).

were tested without successful isolation of the desired recombinant by this technique. No doubt the reason for this stems from the relative size of the homologous regions in common between the plasmid and the chromosome on either side of the insertion site.

The remaining one-third of the *phn*::Tn*phoA'-1* insertions were recombined onto the chromosome by transformation of the *recD phn*(EcoB) host BW17399 with *Nsi*I-digested plasmid DNAs. In most cases, all Kan$^r$ transformants (10 of 10) were the desired type. Two classes of transformants caused some complications, however. One problematic class resulted from transposition of the Tn*phoA'-1* element. These transformants were recognized by their Pn$^+$ phenotype. The other problematic class resulted from transformation with DNAs that were either uncut or recircularized after transformation. These were recognized by their Pn$^+$ and Amp$^r$ phenotypes. Overall, the *recD* technique worked well for all alleles with which it was employed, including the *phnC54* allele, which has only 112 bp of chromosomal DNA on one side of its insertion site. However, only 3 of 53 transformants made with linear DNA from the pWM9*phnC54*::Tn*phoA'-1* plasmid were the desired type.

**Construction of *phn*::Tn*phoA'-1* mutants with a downstream *uidA* fusion.** Once recombined onto the chromosome, each *phn*::Tn*phoA'-1* insertion was transferred by use of P1 into BW17369. This strain is Pn$^+$ and carries the *phn-10*::*uidA-aadA* insertion on the chromosome, in which the *uidA* reporter gene is transcriptionally fused to the 3′ end of the *phnP* gene (19). In this strain, insertions in upstream genes can be tested for polar effects by assaying β-glucuronidase. As expected, the *phn-10*::*uidA-aadA* insertion was lost in 80 to 95% of the transductants because of the close linkage of the *phn*::Tn*phoA'-1* insertions and the *phn-10*::*uidA-aadA* insertion. Transductants that retained the *phn-10*::*uidA-aadA* insertion were saved when introducing *phn*::Tn*phoA'-1* insertions with the *lacZ* reporter gene in the + orientation into BW17369, and transductants that lost the *phn-10*::*uidA-aadA* insertion were saved when introducing *phn*::Tn*phoA'-1* insertions with the *lacZ* reporter gene in the − orientation into BW17369. The latter transductants were used to test for expression of the opposite strand (15). The *uidA-aadA* cassette would be expected to interfere with transcription of the opposite strand if transcription were to initiate at a promoter beyond the 3′ end of the *phnP* gene. The expression of the opposite strand was examined because there are four ORFs on the opposite strand in the *phn* gene cluster (Fig. 1). To be sure that the *phn*::Tn*phoA'-1* insertions had the expected structure and that no rearrangement at the *phn* locus had occurred in these crosses, chromosomal DNAs were examined by DNA hybridization. In all cases, the data were consistent with the structure expected for these mutations on the chromosome (Fig. 6). One unusual recombinant was also examined because it, unexpectedly, showed a Pn$^+$ phenotype. However, it was shown not to have an insertion in the *phn* region and was discarded from further consideration.

**Recombinational switching of *phn*::Tn*phoA'-1* insertions.** The determination of specific roles for each *phn* gene required a method for inactivation of individual genes which took into account effects due to polarity. Since the *phn*::Tn*phoA'-1* insertions were likely to be polar, the mutational effects of these mutations provided little new information on the roles of particular genes. Importantly, Tn*phoA'-1* insertions can be switched to other Tn*phoA'* elements, such as Tn*phoA'-9*, that provide an outward promoter for expression of downstream genes (Fig. 4) (31). Therefore, all + orientation *phn*::Tn*phoA'-1* insertions were switched to Tn*phoA'-9*.

Recombinational switching of *phn*::Tn*phoA'-1* insertions to Tn*phoA'-9* was accomplished in two steps. Each *phn*::Tn*phoA'-1* mutant was first made Tc$^r$ with λ::Tn*phoA-132*. This led to the formation of switched recombinants having *phn*::Tn*phoA-132* or *phn*::Tn*phoA'-2* insertions (Fig. 7A). Ones having *phn*::Tn*phoA'-2* insertions were then made Kan$^r$ with λ::Tn*5-112*. This led to the formation of switched recombinants having *phn*::Tn*phoA'-9* insertions (Fig. 7B). These recombinants were tested for mutational effects and

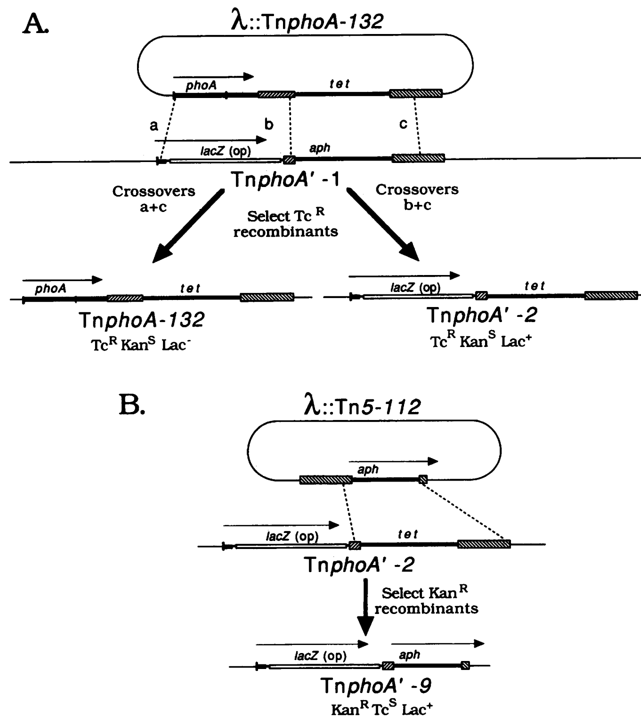


FIG. 7. Recombinational switching of Tn*phoA'* elements. (A) Recombinational switching of *phn*::Tn*phoA'-1* insertions. Tn*phoA* and Tn*phoA'* elements have sufficient DNA in common at both the *phoA* end and the IS*50*R end of each element so that switching of one element to another is efficient (31). Because Tn*phoA'-1* and Tn*phoA-132* also have IS*50*L DNA in common in their central regions, two kinds of switched recombinants were expected. Ones resulting from crossovers a and c were switched for both the drug resistance marker and the type of fusion. Ones resulting from crossovers b and c were switched only for the drug resistance marker. All 23 + orientation *phn*::Tn*phoA'-1* mutants were made $Tc^r$ by using λ::Tn*phoA-132*, and these transductants were tested for their drug resistance and Lac phenotypes. Those having *phn*::Tn*phoA-132* insertions were recognizable as $Kan^s$ $Lac^-$ recombinants, and those having *phn*::Tn*phoA'-2* insertions were recognizable as $Kan^s$ $Lac^+$ recombinants. (B) Recombinational switching of *phn*::Tn*phoA'-2* insertions. Tn*phoA'* and Tn*5-112* elements have sufficient DNA in common within IS*50*L and IS*50*R sequences so that the central region of Tn*5-112* can be switched for the central region of a Tn*phoA'* or a Tn*phoA* element. Also, the IS*50*L and IS*50*R sequences in common are in an arrangement such that one kind of switched recombinant would predominate (31). This kind has the *aph* gene oriented such that an outward promoter would transcribe downstream sequences. All 23 *phn*::Tn*phoA'-2* mutants were made $Kan^r$ by using λ::Tn*5-112*. Recombinants with *phn*::Tn*phoA'-9* insertions were recognized as $Tc^s$ $Lac^+$ recombinants. op, operon.

for expression of the downstream *phn-10*::*uidA* transcriptional fusion as described below.

**Polar effects of *phn*::Tn*phoA'-1* and *phn*::Tn*phoA'-9* insertions.** Two methods were used to assess polar effects due to *phn*::Tn*phoA'* insertions. One method involved measuring β-glucuronidase synthesis in the + orientation mutants during $P_i$ limitation, a condition that leads to induction of the downstream *phn-10*::*uidA* fusion (Table 2). Under this condition, all 23 *phn*::Tn*phoA'-1* mutants made only a low basal level of β-glucuronidase, thus showing that the Tn*phoA'-1* insertions are polar, as expected. These data also showed that no internal *psi* promoter exists between the most

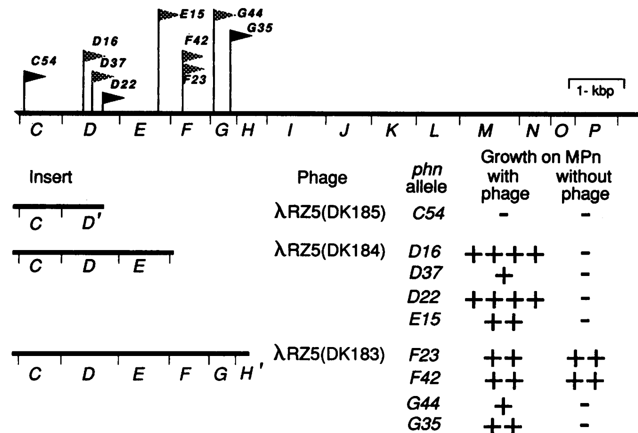


FIG. 8. Complementation of *phn*::Tn*phoA'-9* mutants. Strains carrying Tn*phoA'-9* insertions in genes *phnC*, *phnD*, *phnE*, *phnF*, and *phnG* were lysogenized with a λ*phnC*$^+$, λ*phn*(*CDE*)$^+$, or λ*phn*(*CDEFG*)$^+$ phage. Four lysogens of each mutant were compared with its respective nonlysogenic parental strain for growth on glucose MOPS agar with methylphosphonate (MPn) as the sole P source. The amount of growth relative to that of the wild type is indicated with plus and minus signs; wild-type growth was scored as having four plus signs. See the legend to Fig. 5 for explanation of flags.

upstream insertion, *phnC54*, and the downstream *uidA* reporter gene.

All *phn*::Tn*phoA'-9* mutants except three (the *phnC54*, *phnD37*, and *phnN29* mutants) made at least 2% of the fully induced level of β-glucuronidase. Since the *phnD16*::Tn*phoA'-9* mutant made only 2% of the fully induced level and the phenotype of this mutant was shown not to be due to polarity (see below), this level of expression is apparently sufficient for Pn use. Most mutants made 50 to 150% as much as the wild-type level. The actual amounts were dependent upon the location of the Tn*phoA'-9* insertions. Interestingly, mutants with insertions in the *phnC*, *phnD*, and *phnE* genes made considerably less β-glucuronidase than mutants with insertions in genes further downstream. The low level of β-glucuronidase synthesis in the *phnC*, *phnD*, and *phnE* mutants is consistent with a potential site for down regulation of the *phn* operon near the 3' end of the *phnE* gene (15). Accordingly, down regulation near the *phnE*-to-*phnF* intergenic region may lead to a reduced level of expression of the *phn-10*::*uidA* fusion for transcription initiated from Tn*phoA'-9* insertions upstream of the *phnF* gene. Of the 23 + orientation *phn*::Tn*phoA'-9* mutants, 20 (the *phnD16*, *phnD22*, *phnE15*, *phnF23*, *phnF42*, *phnG44*, *phnG35*, *phnH19*, *phnH13*, *phnH48*, *phnH21*, *phnI40*, *phnJ24*, *phnJ56*, *phnJ14*, *phnL39*, *phnL36*, *phnN32*, *phnN45*, and *phnO38* mutants) showed substantial β-glucuronidase synthesis. Therefore, the mutational effects due to these insertions are not due to polarity.

The availability of λ phages carrying the 5' end of the *phn* gene cluster provided an additional means to test for polar effects due to mutations in the *phnC*, *phnD*, *phnE*, *phnF*, and *phnG* genes (Fig. 8). The ability to complement particular mutants with these phages showed that downstream genes were expressed in those mutants. Conversely, the inability to complement some mutants showed that the corresponding mutations were strongly polar. The *phnD16*::Tn*phoA'-9*, *phnD22*::Tn*phoA'-9*, and *phnE15*::Tn*phoA'-9* mutants were complemented by the *phn*(*CDE*)$^+$ phage λRZ5(DK185).

TABLE 2. Mutational effects of *phn*::Tn*phoA*′-*1* and *phn*::Tn*phoA*′-*9* insertions in + orientation

| *phn* allele | Tn*phoA*′-*1* transductant[a] | β-Glucuronidase sp act[b] | | Growth[c] on: | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | MPn | | Pt | | P-ser | |
| | | Tn*phoA*′-*1* | Tn*phoA*′-*9* | Tn*phoA*′-*1* | Tn*phoA*′-*9* | Tn*phoA*′-*1* | Tn*phoA*′-*9* | Tn*phoA*′-*1* | Tn*phoA*′-*9* |
| *C54* | BW17505 | 0.003 | 0.003 | − | − | − | − | − | − |
| *D16* | BW17479 | 0.003 | 0.008 | − | − | − | − | − | − |
| *D37* | BW17478 | 0.003 | 0.005 | − | − | − | − | − | − |
| *D22* | BW17477 | 0.003 | 0.014 | − | − | − | − | − | − |
| *E15* | BW17467 | 0.003 | 0.007 | − | − | − | − | − | − |
| *F23* | BW17489 | 0.003 | 0.171 | − | ++ | − | + | ++++ | ++++ |
| *F42* | BW17480 | 0.003 | 0.189 | − | ++ | − | + | ++++ | ++++ |
| *G44* | BW17491 | 0.002 | 0.184 | − | − | − | − | ++++ | ++++ |
| *G35* | BW17476 | 0.003 | 0.113 | − | − | − | − | ++++ | ++++ |
| *H19* | BW17497 | 0.002 | 0.089 | − | − | − | − | ++++ | ++++ |
| *H13* | BW17465 | 0.002 | 0.089 | − | − | − | − | ++++ | ++++ |
| *H48* | BW17486 | 0.004 | 0.065 | − | − | − | − | ++++ | ++++ |
| *H21* | BW17474 | 0.003 | 0.229 | − | ++++ | − | ++++ | ++++ | ++++ |
| *I40* | BW17488 | 0.004 | 0.010 | − | − | − | − | ++++ | ++++ |
| *J24* | BW17503 | 0.003 | 0.060 | − | − | − | − | ++++ | ++++ |
| *J56* | BW17500 | 0.004 | 0.504 | − | − | − | − | ++++ | ++++ |
| *J14* | BW17466 | 0.002 | 0.290 | − | − | − | − | ++++ | ++++ |
| *L39* | BW17498 | 0.003 | 0.181 | − | − | − | − | ++++ | ++++ |
| *L36* | BW17499 | 0.003 | 0.176 | − | − | − | − | ++++ | ++++ |
| *N29* | BW17553 | 0.003 | 0.004 | ++ | ++ | ++ | ++ | ++++ | ++++ |
| *N32* | BW17581 | 0.003 | 0.366 | ++ | ++ | ++ | ++ | ++++ | ++++ |
| *N45* | BW17427 | 0.003 | 0.579 | ++ | ++ | ++ | ++ | ++++ | ++++ |
| *O38* | BW17436 | 0.005 | 0.181 | − | +++ | − | ++ | ++++ | ++++ |

[a] The wild-type strain was BW17369. The *phn*::Tn*phoA*′-*1* insertions were crossed into BW17369 by P1 transduction using P1*kc* grown on recombinants carrying the respective insertion on the chromosome. Each insertion was recombined onto the chromosome by conjugative transfer of pWM9*phn*::Tn*phoA*′-*1* or pWM10*phn*::Tn*phoA*′-*1* plasmid into BW17204 and subsequent selection for $Tc^s$ recombinants or by transformation of BW17399 with *Nsi*I-cut plasmid DNAs. Each BW17369*phn*::Tn*phoA*′-*1* transductant was switched to a *phn*::Tn*phoA*′-*2* mutant with λ::Tn*phoA*-*132* by selection for $Tc^r$ transductants. $Kan^s$ $Lac^+$ and $Kan^s$ $Lac^-$ recombinants from these crosses contain *phn*::Tn*phoA*′-*2* and *phn*::Tn*phoA*-*132* insertions, respectively (Fig. 7A). Each *phn*::Tn*phoA*′-*2* transductant was then switched to a *phn*::Tn*phoA*′-*9* mutant with λ::Tn*5*-*112* by selection for $Kan^r$ transductants. $Tc^s$ recombinants from these crosses contain *phn*::Tn*phoA*′-*9* insertions (Fig. 7B).

[b] β-Glucuronidase was measured in $P_i$-limited cultures as described in Materials and Methods. The specific activity of the wild type was 0.400.

[c] Growth was tested on glucose MOPS agar with methylphosphonate (MPn), phosphite (Pt), or phosphoserine (P-ser) as the sole P source, as described in Materials and Methods, and scored as wild-type growth (++++), intermediate growth (+++, ++, or +), or no growth (−).

Therefore, mutational effects due to these mutations are not due to polarity. In agreement, these insertions were not strongly polar on expression of the *phn-10*::*uidA* fusion. The *phnC54*::Tn*phoA*′-*9* mutant was not complemented by the $phnC^+$ phage λRZ5(DK184), and the *phnD37*::Tn*phoA*′-*9* mutant was poorly complemented by the $phn(CDE)^+$ phage. Therefore, these mutations clearly cause polar effects. In agreement, these insertions were strongly polar on the *phn-10*::*uidA* fusion (Table 2). In addition, *phnF* and *phnG* mutants were tested for complementation by the $phn$ $(CDEFG)^+$ phage λRZ5(DK183). Both *phnF*::Tn*phoA*′-*9* mutants, which are identical, showed a similar $Pn^+$ phenotype with or without the phage. These results are consistent with a regulatory role for the PhnF protein. The *phnG44*::Tn*phoA*′-*9* mutant was poorly complemented, and the *phnG35*::Tn*phoA*′-*9* mutant was partially complemented, by this phage. The partial complementation of the *phnG* mutants corroborates the evidence that the *phnG* product is required for Pn use. It is not understood why *phnG* mutants are not fully complemented, because neither *phnG* mutation was strongly polar on the *phn-10*::*uidA* fusion. Since the $\lambda phn(CDEFG)^+$ phage carries the 5′ end of the *phnH* gene, the failure to see full complementation with this phage may be due to interference by a truncated *phnH* gene product in these merodiploid mutants.

**Mutational effects of *phn*::Tn*phoA*′ insertions.** It was previously shown that $Pn^-$ mutants were also phosphite negative, presumably because a common function is required for Pn and phosphite use. In addition, it was shown that certain $Pn^-$ mutants, which are believed to be blocked in uptake, were also phosphoserine negative if the mutant was *phoA*. Since phosphoserine use by a *phoA* mutant is dependent upon a cytoplasmic phosphatase, the Pn transporter apparently also transports phosphoserine (17). Therefore, the *phn*::Tn*phoA*′-*1* and *phn*::Tn*phoA*′-*9* insertions were also tested for effects on the use of these alternative P sources.

All 43 *phn*::Tn*phoA*′-*1* insertions affected Pn use when recombined onto the chromosome. Also, these mutations had similar effects with respect to Pn and phosphite use. The 39 $Pn^-$ mutants were also phosphite negative; the 4 weakly $Pn^+$ mutants were also weakly phosphite positive (Tables 2 and 3). The latter were *phnN* mutants. In contrast, mutants were distinguishable in regard to their use of phosphoserine. Only 10 mutants were phosphoserine negative; these had insertions in the *phnC*, *phnD*, or *phnE* gene. Thirty-three mutants were phosphoserine positive; these had insertions in a downstream gene. The phosphoserine-negative mutants are probably defective in uptake, and the phosphoserine-positive mutants are not defective in uptake. The latter are apparently defective in cleavage of the C-P bond.

The mutational effects of 23 + orientation mutations were compared as *phn*::Tn*phoA*′-*1* and *phn*::Tn*phoA*′-*9* derivatives to determine whether effects were due to polarity. Of the 20 *phn*::Tn*phoA*′-*9* insertions that were not strongly polar, 14 led to a $Pn^-$ phenotype, including the *phnD16*, *phnD22*, *phnE15*, *phnG44*, *phnG35*, *phnH19*, *phnH13*,

TABLE 3. Mutational effects of *phn*::Tn*phoA'-1* insertions in the − orientation

| *phn* allele | Strain[b] | Growth[c] on: | | |
|---|---|---|---|---|
| | | MPn | Pt | P-ser |
| WT[a] | BW17369 | ++++ | ++++ | ++++ |
| *C27* | BW17493 | − | − | − |
| *D46* | BW17492 | − | − | − |
| *D53* | BW17484 | − | − | − |
| *D47* | BW17468 | − | − | − |
| *E18* | BW17469 | − | − | − |
| *F20* | BW17470 | − | − | ++++ |
| *G50* | BW17481 | − | − | ++++ |
| *I26* | BW17475 | − | − | ++++ |
| *I43* | BW17490 | − | − | ++++ |
| *K52* | BW17483 | − | − | ++++ |
| *K37* | BW17472 | − | − | ++++ |
| *L51* | BW17482 | − | − | ++++ |
| *L30* | BW17473 | − | − | ++++ |
| *M28* | BW17471 | − | − | ++++ |
| *N34* | BW17496 | ++ | ++ | ++++ |
| *O17* | BW17495 | − | − | ++++ |
| *O49* | BW17487 | − | − | ++++ |
| *P55* | BW17509 | − | − | ++++ |
| *P57* | BW17485 | − | − | ++++ |
| *P25* | BW17494 | − | − | ++++ |

[a] WT, wild type.

[b] Mutations were crossed into BW17369 by using P1*kc* grown on recombinants carrying the respective *phn*::Tn*phoA'-1* insertion on the chromosome, by selection for Kan[r] transductants. Insertions were recombined onto the chromosome as described in footnote *a* of Table 2.

[c] Growth was assessed as for Table 2.

*phnH48*, *phnI40*, *phnJ24*, *phnJ56*, *phnJ14*, *phnL39*, and *phnL36* alleles (Table 2). Since such nonpolar *phnD* and *phnE* mutants were phosphoserine negative, the PhnD and PhnE proteins are required for uptake. Since such nonpolar *phnG*, *phnH*, *phnI*, *phnJ*, and *phnL* mutants were phosphoserine positive, the PhnG, PhnH, PhnI, PhnJ, and PhnL proteins are required for catalysis. In spite of the Pn$^+$ phenotype of the *phnH21*::Tn*phoA'-9* mutant, the PhnH protein is probably required because three other *phnH* mutants (the *phnH19*, *phnH13*, and *phnH48* mutants) were Pn$^-$. The *phnH21*::Tn*phoA'-9* insertion may not cause a Pn$^-$ phenotype because it is near the 3′ end of the *phnH* gene. Three other mutants (the *phnF23*, *phnF42*, and *phnO38* mutants) were Pn$^-$ as Tn*phoA'-1* derivatives and were fully or partially Pn$^+$ as Tn*phoA'-9* derivatives. The effects of these Tn*phoA'-1* insertions were therefore due to polarity, and the PhnF and PhnO proteins are not required for Pn use. In agreement, a nonpolar *phnO5*::Tn*5-112* mutant was previously shown to be Pn$^+$ (17).

The other two strongly polar mutants had insertions in the *phnN* gene (the *phnN32* and *phnN45* mutants). Interestingly, all *phnN* mutants had a weak Pn$^+$ phenotype, as either Tn*phoA'-1* or Tn*phoA'-9* derivatives. Two (the *phnN32*::Tn*phoA'-1* and *phnN34*::Tn*phoA'-1* mutants) were identified as Pn$^-$ plasmid mutants, one (the *phnN29*::Tn*phoA'-1* mutant) was identified as a weak Pn$^+$ plasmid mutant, and one (the *phnN45*::Tn*phoA'-1* mutant) was identified as a Pn$^+$ Psi-LacZ$^+$ plasmid mutant. Three (the *phnN29*::Tn*phoA'-1*, *phnN32*::Tn*phoA'-1*, and *phnN45*::Tn*phoA'-1* mutants) are in the + orientation and one (the *phnN34*::Tn*phoA'-1* mutant) is in the − orientation. Since all four *phnN* insertions led to a weak Pn$^+$ phenotype as chromosomal mutations, the PhnN protein apparently does not have an absolute role in Pn use. It is not understood why the phenotypes were different for certain plasmid and chromosomal *phnN* mutations, however. Interestingly, the *phnN29*::Tn*phoA'-1*, *phnN329*::Tn*phoA'-1*, *phnN29*::Tn*phoA'-1*, and *phnN29*::Tn*phoA'-9* insertions were strongly polar on the *phn-10*::*uidA* fusion and thus do not express the *phnP* gene. Therefore, *phnN* mutants may be partially relieved of the requirement for the *phnP* gene product. The *phnP*, but not the *phnO*, gene product was shown to be required for Pn use in *phnN*$^+$ strains (17, 19; this paper).

All 20 − orientation insertions except one (*phnN34*) caused a Pn$^-$ phenotype (Table 3). Only four of these were informative, however. The Pn$^-$ phenotype due to the *phnM28* insertion showed that the PhnM protein is required for Pn use. If the *phnM* mutant phenotype were instead due to a polar effect, then the *phnM* insertion would be expected to cause a weak Pn$^+$ phenotype, like *phnN* insertions. The Pn$^-$ phenotype due to the *phnP* insertions (*phnP55*, *phnP57*, and *phnP25*) provides further evidence that the PhnP protein has a role in Pn use. Previously, the *phnP2*::Tn*5-112* and *phnP3*::Tn*5-112* mutants (17), as well as the Δ*phnP11* mutant (19), were shown to be Pn$^-$.

## DISCUSSION

The *phn* locus was originally called *psiD* because $P_i$-regulated *psiD*::*lacZ* fusions made with Mu d1 abolished the ability to use Pn as a P source (24). Unexpectedly, the minimal-size DNA fragment needed for complementation of such mutants proved to be quite large (29). Consequently, the entire 15.6-kbp insert of one Pn$^+$ plasmid was sequenced (7). On the basis of the DNA sequence, it was proposed that the *phn* (*psiD*) locus was an operon of 17 genes, named in alphabetical order *phnA* to *phnQ*. Subsequently, it was shown that the *phnA* and *phnB* ORFs were not required for Pn use (19) and that a *psi* promoter immediately preceded the *phnC* gene (12, 19). Also, the putative *phnQ* ORF was shown to be artifactual by using plasmids with *phnQ* insertion mutations (19; this paper).

Two functions were assigned to the *phn* gene cluster on the basis of the predicted protein sequences (7). It was thought to encode an uptake system because the PhnC, PhnK, and PhnL proteins have sequence similarities to the ATP-dependent permease component of binding protein-dependent transport systems and because the PhnM protein has sequence similarities to the integral membrane component of such transporters. It was assumed that many of the remaining *phn* gene products constituted a C-P lyase enzyme complex. In addition, the PhnL and PhnO proteins were considered candidates for regulatory proteins because each contains a helix-turn-helix motif. Also, the PhnE protein was identified as a probable membrane protein, although no function was assigned to PhnE.

Mutational studies provided evidence for three functions because phenotypic tests allowed distinguishing of different classes of mutations (17). One class abolished Pn use, and when tested in a *phoA* host, this class also abolished phosphoserine use. Such mutations probably affect an uptake system because phosphoserine use by a *phoA* mutant is dependent upon a cytoplasmic phosphatase. Another class abolished only Pn use, as these mutations had no effect on phosphoserine use. Such mutations probably alter a component of the C-P lyase since they cause a Pn$^-$ phenotype and do not affect uptake. A third class caused no phenotypic effect except those attributable to polarity. Mutations in this class may alter a nonessential regulatory protein.

Transposon mutagenesis of the *phnCDEFGHIJKLMNOP*

gene cluster was carried out by using Tn*phoA′-1* because this element causes polar mutations and would therefore cause more $Pn^-$ mutants. The characterization of the *phnC54* insertion showed that no internal *psi* promoter exists in this gene cluster and that this gene cluster is therefore transcribed as a 14-gene operon. The *phnC54* mutation is only 190 bp downstream from the *phnC* promoter, yet it abolished expression of the 3′ end of the *phnP* gene because of polarity.

Altogether, three to six different mutations were identified in most *phn* genes. Only two *phnC* mutations and one *phnM* mutation were identified. Also, two of the three *phnF* mutations were at the same site (Fig. 5). All *phn*::Tn*phoA′-1* insertions were recombined onto the chromosome in a single copy to test for mutational effects. These insertions were then switched to Tn*phoA′-9*, which provides an outward promoter for downstream gene expression, and polar effects were judged by measurements of β-glucuronidase from a downstream *uidA* reporter gene (Fig. 7). The *phn*::Tn*phoA′-9* insertions in the *phnCDEFG* region were also tested for polar effects by testing for complementation by phages carrying this region. The results allowed for determination of the function of 13 of the 14 *phn* genes, as discussed below.

The PhnC, PhnD, and PhnE proteins probably constitute a binding protein-dependent transport system, in which PhnC is the permease component, PhnD is the periplasmic binding protein, and PhnE is the integral membrane protein. That the *phnCDE* genes alone encode a transporter was shown by complementation of a Δ*phn* Δ*phoA* mutant, using the λ*phn(CDE)*$^+$ phage for growth on phosphoserine (data not shown). Also, mutations that are not strongly polar in the *phnD* and *phnE* genes abolish phosphoserine use in a *phoA* host. Although no nonpolar mutation in the *phnC* gene was found, the PhnC protein is probably the permease component because it has striking sequence similarity to permease components of such transport systems. The PhnD protein is almost certainly the binding protein because it has features of a binding protein. The PhnD protein is a hydrophilic protein with characteristics of a signal peptide at its N terminus. Furthermore, the PhnD protein is probably localized to the periplasm because a *phnD*::Tn*phoA* mutant with an in-frame gene fusion produces an active alkaline phosphatase (15). The PhnE protein is probably the integral membrane component because it is a very hydrophobic protein. Also, the PhnE protein has some sequence similarity to the integral membrane components RbsC and PstA of binding protein-dependent transport systems for ribose and $P_i$, respectively (data not shown).

The PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, and PhnM proteins are probably components of a membrane-associated C-P lyase enzyme complex. That the C-P lyase is likely a membrane-associated enzyme complex is inferred from the hydrophobic nature of the 42-kDa *phnM* gene product and from the large number of required proteins. Furthermore, a membrane-associated enzyme complex is consistent with the hydrophobic nature of the PhnM protein and the proposed involvement of redox chemistry in C-P bond cleavage by a lyase (17).

Two additional proteins, PhnN and PhnP, may be accessory proteins for the C-P lyase. PhnN is likely an accessory protein because *phnN* mutants showed a weak $Pn^+$ phenotype. An accessory role for PhnP is inferred because polar *phnN* mutations probably abolished expression of the *phnP* gene. Since *phnP* insertion mutants are $Pn^-$, PhnP is apparently required only in the presence of a functional *phnN* gene product. Alternatively, it is conceivable that PhnN is a regulatory protein that is required for expression of the *phnP* gene. However, this possibility seems unlikely because if this were the case, *phnN* mutations that are not strongly polar would have been expected to be $Pn^+$. Such mutations also led to a weak $Pn^+$ phenotype. It is also unlikely that only a small amount of the *phnP* gene product suffices because polar, but not nonpolar, insertions in the *phnO* gene cause a $Pn^-$ phenotype (17).

Both the PhnF and PhnO proteins may be regulatory proteins. The primary effects of *phnF* and *phnO* mutations are attributable to polarity. In addition, the PhnF protein may be a member of a new family of regulatory proteins together with the FadR, GntR, HutC, KorA, GenA, and P30 proteins (9). These proteins share a highly conserved N-terminal domain that has the potential to form a helix-turn-helix DNA binding motif. Furthermore, some members of the putative PhnF gene family appear to contain a consensus regulatory site, which closely matches a sequence in the 5′ end of the *phnF* structural gene. This site could be important for down regulation of the *phnCDEFGHIJKLMNOP* operon (15). Alternatively, the PhnF protein may be an accessory protein for the C-P lyase, since nonpolar *phnF* mutations showed an intermediate level of growth on Pn. The evidence that the PhnO protein is a regulatory protein is less convincing, as the PhnO protein is not similar to other regulators and no studies on the PhnO protein have been done. Although the PhnO protein contains a potential helix-turn-helix motif, the primary reason for proposing a regulatory role for the PhnO protein is the absence of an effect of nonpolar *phnO* mutations on Pn use.

In summary, the *phn* gene cluster is transcribed as the 14-gene, 10.9-kbp *phnCDEFGHIJKLMNOP* operon. The *phnCDE* genes probably encode a binding protein-dependent Pn transport system; the *phnGHIJKLM* genes probably encode a C-P lyase that has two accessory proteins, the *phnN* and *phnP* gene products; and the *phnF* and *phnO* genes may encode regulatory proteins. Also, the C-P lyase is probably a membrane-associated enzyme complex, which could explain the inability to detect an activity in cell extracts. Furthermore, the mechanism for C-P bond cleavage by the C-P lyase probably involves redox chemistry because all $Pn^-$ mutations are also phosphite negative. Importantly, in vivo studies have shown that breakage of the C-P bond by the *E. coli* lyase leads to racemization at the reacting carbon center (3), which is compatible with a mechanism involving either radical or redox chemistry.

In addition, it should be pointed out that the P product of the C-P lyase has not been established. The P product of the lyase does not have to be $P_i$ for Pn to serve as a P source. In this regard, it is especially tantalizing that two components of the lyase, the PhnK and PhnL proteins, have sequence similarities to ATP-dependent permeases. These similarities may indicate a role for ATP, or another nucleotide, in catalysis. Importantly, the direct transfer of a phosphoryl group from a Pn to a nucleotide by the C-P lyase would allow for Pn use as a P source, without the release of $P_i$. That C-P bond cleavage by a lyase may involve the formation of a nucleotide intermediate is consistent with the generation of a ribosyl-phosphonate derivative during Pn degradation in vivo (5). Furthermore, this is compatible with the physiological behavior of bacteria during growth on Pn. The PHO regulon is fully derepressed during growth on a Pn broken down by a C-P lyase, thus showing that (excess) $P_i$ is not released. In contrast, the PHO regulon is only partially derepressed during growth on a Pn broken down by the

phosphonatase pathway, which releases $P_i$ as a product of C-P bond cleavage (11).

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