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Involvement of the *Escherichia coli phn* (*psiD*) Gene Cluster in Assimilation of Phosphorus in the Form of Phosphonates, Phosphite, P_i Esters, and P_i

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The *phn* (*psiD*) gene cluster is induced during P_i limitation and is required for the use of phosphonates (Pn) as a phosphorus (P) source. Twelve independent Pn-negative (Pn^-) mutants have lesions in the *phn* gene cluster which, as determined on the basis of recombination frequencies, is larger than 10 kbp. This distance formed the basis for determining the complete DNA sequence of a 15.6-kbp *Bam*HI fragment, the sequences of which suggested an operon with 17 open reading frames, denoted (in alphabetical order) the *phnA* to *phnQ* genes (C.-M. Chen, Q.-Z. Ye, Z. Zhu, B. L. Wanner, and C. T. Walsh, J. Biol. Chem. 265:4461–4471, 1990) Ten Pn^- lesions lie in the *phnD*, *phnE*, *phnH*, *phnJ*, *phnK*, *phnO*, and *phnP* genes. We propose a smaller gene cluster with 14 open reading frames, *phnC* to *phnP*, which probably encode transporter and regulatory functions, in addition to proteins needed in Pn biodegradation. On the basis of the effects on phosphite (Pt), P_i ester, and P_i use, we propose that PhnC, PhnD, and PhnE constitute a binding protein-dependent Pn transporter which also transports Pt, P_i esters, and P_i. We propose that PhnO has a regulatory role because a *phnO* lesion affects no biochemical function, except for those due to polarity. Presumably, the 10 other *phn* gene products mostly act in an enzyme complex needed for breaking the stable carbon-phosphorus bond. Interestingly, all Pn^- mutations abolish the use not only of Pn but also of Pt, in which P is in the +3 oxidation state. Therefore, Pn metabolism and Pt metabolism are related, supporting a biochemical mechanism for carbon-phosphorus bond cleavage which involves redox chemistry at the P center. Furthermore, our discovery of P_i-regulated genes for the assimilation of reduced P suggests that a P redox cycle may be important in biology.

Living systems satisfy their need for phosphorus (P) primarily through P_i, in which P is in its highest, +5, oxidation state. P_i enters *Escherichia coli* via the low-affinity P_i transporter (Pit) when present in excess or via the high-affinity phosphate-specific transporter (Pst) when present in low levels ($<4 \times 10^{-6}$ M). The low-affinity P_i transporter is a single-component system, analogous to LacY, which is made constitutively (41). In contrast, Pst is a multicomponent periplasmic binding protein-dependent system (16) encoded by the *pstSCAB-phoU* operon, the expression of which is highly regulated. The *pstS* promoter is expressed at a low, basal level when P_i is present in excess; it shows 100-fold derepression during P_i-limited growth (unpublished data).

While P_i is the preferred P source, *E. coli* also uses organophosphates as a sole P source. However, P_i esters in general do not enter cells intact. Many esters are instead degraded in the periplasm, allowing the released P_i to be taken up via the low-affinity P_i transporter or Pst system. Once inside the cell, P_i is captured in one of several metabolic reactions which require energy and produce organophosphate esters. Although many steps in metabolism produce or consume P_i, a key feature of genes or operons with primary roles in P assimilation is their induction during P_i limitation (50, 52).

The phosphate regulon (PHO regulon) consists of several P_i starvation-inducible (*psi*) genes, the products of which act primarily in the assimilation of environmental P (52). The PHO regulon includes (i) the *phoBR* operon, which encodes the environmental P_i sensor, PhoR, and the PHO regulon activator, PhoB (29, 30); (ii) the aforementioned *pstSCAB-phoU* operon; (iii) the *phoA* operon (34), which encodes bacterial alkaline phosphatase (Bap), a nonspecific phosphomonoesterase; (iv) the *phoE* gene, which encodes an outer membrane porin selective for polyanions (5); (v) the *ugpBAEC* transporter for glycerol 3-phosphate (39) which, like Pst, encodes a binding protein-dependent system; and (vi) the *phnC* to *phnP* gene cluster, the subject of this paper.

Since Bap is localized to the periplasm, its increased synthesis during P_i limitation allows *E. coli* to use many small, nontransportable P_i esters as sole P sources. Also, large phosphorylated compounds may serve as substrates for Bap. Many such compounds probably gain access to the periplasm through the PhoE porin, which allows the passage of complex anions across the outer membrane. Although relatively few P_i esters are thought to enter cells intact, the *ugpBAEC* transporter (9) apparently has a role in the entry of P_i esters. In this regard, Ugp is the only previously reported transporter for a P_i ester the synthesis of which is P_i regulated and under PhoB and PhoR control. (Although GlpT and UhpT transport glycerol 3-phosphate and hexose 6-phosphates, respectively, for the use of the respective esters as C and energy sources [19, 21], neither GlpT synthesis nor UhpT synthesis is P_i regulated.)

Phosphonates (Pn) are similar to P_i esters, except that Pn have direct carbon-phosphorus (C-P) bonds in place of the carbon-oxygen-phosphorus ester linkage. Also, like P_i esters, Pn are widespread in nature. Pn are commonly found in the form of phosphonolipids. Although they exist in organisms from bacteria to mammals, their biological role is unknown (26). Unlike P_i ester bonds, which are readily

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hydrolyzed, C-P bonds are extremely stable. Hence, breaking the C-P bond is biochemically difficult. As nonhydrolyzable P_i ester analogs, Pn are used as enzyme inhibitors, as antibacterial, antiviral, and antitumor agents, and as herbicides. Thus, both their biosynthesis and their biodegradation are of interest. The biodegradation of Pn, which allows for their use as a P source, requires cleavage of the C-P bond.

Both gram-positive and gram-negative bacteria which use Pn as a sole P source and can therefore break the C-P bond have been isolated (42). Two metabolic pathways exist for Pn biodegradation. In one pathway, Pn with a substituted 2-carbon, such as aminoethylphosphonate (AEPn) and phosphonoacetate (PnAc), are degraded via transamination-mediated dephosphonation, in which the last step is hydrolytic cleavage by phosphonoacetaldehyde phosphohydrolase (trivial name, phosphonatase [38]). However, Pn with an unsubstituted 2-carbon, such as ethylphosphonate (EPn) and methylphosphonate (MPn), cannot be degraded via the phosphonatase pathway because the carbon products, as determined in in vivo studies, are the corresponding hydrocarbons (15, 45). Instead, they are degraded via a different pathway, which is more common in gram-negative bacteria. In the alternative pathway, fission of the C-P bond apparently involves direct dephosphonation by an enzyme for which the trivial name C-P lyase was adopted (45). In vivo evidence for C-P lyase activity is provided by the production of the hydrocarbons ethane and methane, respectively, as by-products when Pn-positive (Pn^+) *E. coli* is grown on media with EPn and MPn as the sole P sources (3, 46). However, despite numerous attempts, it has been difficult to detect C-P lyase activity in cell extracts. Hence, the biochemical mechanism of C-P lyase remains poorly understood.

The *phn* gene cluster has a role in P assimilation because its expression is induced during P_i limitation in a manner that requires the PHO regulon regulatory proteins, PhoB and PhoR (54). The discovery that *E. coli phn* (*psiD*) mutants did not use Pn as a sole P source led to the suggestion that the *phn* gene cluster encodes C-P lyase (46). Although wild-type *E. coli* K-12 cannot use Pn as a sole P source, it readily yields mutants which can. Such mutants arise by activation of the cryptic *phn*($EcoK^0$) allele. Despite the cryptic nature of the *phn*($EcoK^0$) locus in *E. coli* K-12, most *E. coli* strains, including *E. coli* B, are naturally Pn^+ (53).

In the absence of a biochemical assay, genetic studies on Pn metabolism were undertaken in *E. coli* (53; this paper). First, both the cryptic *phn*($EcoK^0$) allele and mutations for its activation were mapped near 92.8 min. Second, *phnD*::*lacZ*(Mu d1) insertions, which were identified as phosphate-regulated *lacZ* transcriptional fusions (54), abolished mutatability to Pn^+. Third, *E. coli* K-12 hybrids with the *phn* (EcoB) allele are naturally Pn^+. Fourth, all *phn* mutations simultaneously abolished the use of all Pn tested as sole P sources, including the natural Pn AEPn as well as the synthetic Pn EPn, MPn, and PnAc (53; this paper). In addition, certain *phn* mutations abolished the use of P_i esters in a *phoA*-independent pathway and of P_i in a pathway that bypasses the Pst transporter. This result suggests that the *phn* gene cluster may encode a Pn transporter which also transports P_i esters and P_i.

E. coli also uses phosphite (Pt), in which P is in the +3 oxidation state (10, 31), as a sole P source. Importantly, all *phn* mutations simultaneously abolish the use of both Pn and Pt. Hence, Pn and Pt metabolism are related via common mechanisms for both transport and biodegradation. Our results are compatible with a mechanism for C-P bond fission that involves reductive cleavage of the C-P bond and a Pt intermediate. This is the first genetic evidence for a P redox cycle in nature.

MATERIALS AND METHODS

Nomenclature of *phn* alleles. The *phn* loci differ in *E. coli* K-12 and B strains and are denoted *phn*($EcoK^0$) and *phn* (EcoB), respectively. The *phn*($EcoK^0$) locus is cryptic, while the *phn*(EcoB) locus is naturally Pn^+ (53). An activated *phn*($EcoK^0$) locus is denoted *phn*($EcoK^+$).

Media and chemicals. In general, the media and chemicals were the same as those reported previously (49). P compounds were filter sterilized and added to glucose-morpholine propanesulfonic acid (MOPS) media at 0.1 mM to test for use. PnAc was from Alfa (Danvers, Mass.) and was purified by L. Wackett for use in this work; *o*-phosphoserine was from Sigma Chemical Co. (St. Louis, Mo.); and phosphorous acid was from Aldrich (Milwaukee, Wis.). 5-Bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (X-P_i) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were from Bachem Fine Chemicals (Torrance, Calif.) and are the blues dyes for detecting Bap and β-galactosidase, respectively. X-P_i was used at 0.1 mM as a sole P source and at 40 μg/ml as an indicator in glucose-MOPS-P_i agar. Benzylviologen, dehydroproline, and 2,3,5-triphenyltetrazolium hydrochloride were from Sigma. Tetracycline-sensitive (Tc^s) mutants were selected as described previously (8).

Bacteria, phages, and plasmids. Bacteria are described in Table 1, Table 2, or below. CE77 with Tn*5-112* (6) was from D. Berg; EM41 was from S. Maloy; LS205 and LS206 were from C. Manoil; M72 (*gal-3* ΔH1; 11) was from M. Ptashne; M9s (40) was from A. Böck; MPh2 was from D. Boyd; RB791, SL724, SL1158, SL7910, SL7911, and pSL263 (3, 28) were from J. Frost; RM323 was from R. Maurer; and YMC9 harboring pRK608 with Tn*5-235* (18) was from G. Walker. BW13908 is a P1 transductant with ΔH1 (λ *N7 N53* c*I857* H1 *bio chl*) from M72 and was used as an isogenic formate dehydrogenase-negative control strain. *chl* mutants are formate dehydrogenase negative because of the absence of the molybdenum cofactor.

λ::Tn*5*seq1 (36; λ c*I857 b*221 *P*am80) was from D. Berg. λ::Tn*5-132* (λ c*I857 b*221 *O*am29 *P*am80 *rex*::Tn*5-132*) was described previously (34). All phages were plaque purified to prepare lysates. λ *O*am or *P*am lysates were made on BW10168, a *lac-169* transductant of LE392. Tn*5-112* and Tn*5-235* were crossed onto a λ vector from ColE1::Tn*5-112* in CE77 and pRK608 (pRS2013::Tn*9*::Tn*5-235*), respectively. λ::Tn*5-112* and λ::Tn*5-235* were made by growing λ::Tn*5-132* on CE77 or a pRK608 transformant of BW10168 to make mixed lysates containing λ::Tn*5-112* and λ::Tn*5-235* recombinant phages, respectively. To purify the recombinant phages from the mixed lysates, we selected λ::Tn*5-112* transductants as kanamycin-resistant (Kan^r) dilysogens of BW10204 at 30°C; dilysogens were heat induced, and the resultant lysates were plated on BW10168 on X-Gal indicator agar to identify colorless (Lac^-) λ::Tn*5-112* plaques. λ::Tn*5-235* phages were isolated directly from the original mixed lysate as phages that made blue (Lac^+) plaques on BW10168. λ::Tn*5-112* and λ::Tn*5-235* were verified by their ability to produce Kan^r Tc^s recombinants of the *phn*::Tn*5-132* mutants.

Growth and phenotypic testing of mutants. Plate tests for P use were done by streaking cells on glucose-MOPS agar (purified agar; Difco Laboratories, Detroit, Mich.) with each compound as the sole P source. Suitable positive and nega-

TABLE 1. Bacterial strains

Strain[a]	Genotype or phenotype	Pedigree	Source
BW3372	*proC*::Tn*5* phoR68	BD792 (49)	Kan^r with P1 on BW574 (49)
BW4822	*lac-169* Δ(*psiF proC aroLM phoBR*)*9-6 tsx-234*::Tn*10 creB510 thi*	XPhla via BW3215 (49)	Tc^r with P1 on P2595 (47)
BW4884	*phoR68*	BD792 via BW3372	Spontaneous Pro$^+$
BW4974	*lac-160* Δ(*pstCAB phoU bglCSB*)*201* P_i-*phn*(EcoK^0)	BD792 via BW3850 (48)	Ilv$^+$ with P1 on AW72 (49)
BW7969	*lac-169* DE10(*phoA8*) *creB510 thi*	XPhla via BW6313 (1)	Pro$^+$ with P1 on BW7936 (1)
BW9115	*lac-169* Δ(*psiF proC aroLM phoBR*)*9-6 tsx-234*::Tn*10 creB510 supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 lacY1 tonA*	LE392 (51)	Tc^r with P1 on BW4822
BW9998	*lac-169 phoR68 proC*::Tn*5-132*	BD792 via BW3369 (47)	Tc^r Kan^s swap with λ::Tn*5-132*
BW10168	*lac-169 creB510 supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA*	LE392 via BW9115	Pro$^+$ with P1 on BW3912 (49)
BW10204	λ gt11 *P*am3 *lac-169 creB510 supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA*	LE392 via BW10168	Amp^r with λ gt11 *P*am3 in RM323
BW10535	*serB*::Tn*5 phoR68*	BD792 via BW4884	Kan^r with P1 on CBK018 (1)
BW10735	*serB*::Tn*5-132 phoR68*	BD792 via BW10535	Tc^r Kan^s swap with λ::Tn*5-132*
BW10748	*lac-169 phn*(EcoB) *creB510 hsdR514*	BD792 via BW10733	53
BW11331	DE3(*lac*)X74	$MG1655_{MC}$ via BW10244	53
BW11551	*lac-169* Δ(*phoBR*)*525*	$MG1655_{MC}$ via BW10244	Pro$^+$ with P1 on MPh2
BW12069	DE3(*lac*)X74 Δ*phoA532*	$MG1655_{MC}$ via BW10244	Pro$^+$ with P1 on SM796 (1)
BW12348	*lac-169 serB*::Tn*5-132* DE10(*phoA8*) *creB510 thi*	XPhla via BW7969	Tc^r with P1 on BW10735
BW12350	*lac-169 serB*::Tn*5-132 phn*(EcoK$^+$) DE10(*phoA8*) *creB510 thi*	XPhla via BW7969	Pn$^+$ then Tc^r with P1 on BW10735
BW12655	DE3(*lac*)X74 *zjd-1*::Tn*10* Δ(*phnD-phnP*)*33-30* Δ*phoA532*	$MG1655_{MC}$ via BW12069	Tc^r with P1 on BW10197 (53)
BW12707	*lac-169 proC*::Tn*5-132* Δ(*phoBR*)*525*	$MG1655_{MC}$ via BW11551	Tc^r with P1 on BW9998 (34)
BW12720	DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532*	$MG1655_{MC}$ via BW12655	Pn$^+$ with P1 on BW10748
BW12808[b]	*lac-169* Δ(*phoBR*)*1/527*::Tn*5*(SacII) *creB510 rpsL267 thi*	XPhla via BW12187	Tc^s
BW12856	*lac-169* Δ*phoA532* Δ(*phoBR*)*525*	$MG1655_{MC}$ via BW12707	Pro$^+$ with P1 on SM796 (1)
BW13034	Δ*phoA532* Δ(*phoBR*)*525*	$MG1655_{MC}$ via BW10244	Pro$^+$ with P1 on BW12856
BW13183	DE3(*lac*)X74 *proC*::Tn*5-132*	$MG1655_{MC}$ via BW11331	Tc^r with P1 on BW10244
BW13207	*lac-169 phnD31*::*lacZ*(Mu d1-1734) *phoU35 creB510 thi*	XPhla via BW6504 (49)	Kan^r with P1 on BW12057 (53)
BW13208	*lac-169 phnD33*::*lacZ*(Mu d1-1734) *phoU35 creB510 thi*	XPhla via BW6504	53
BW13209	*lac-169 phnD41*::*lacZ*(Mu d1-1734) *phoU35 creB510 thi*	XPhla via BW6504	Kan^r with P1 on BW12059 (53)
BW13247	DE3(*lac*)X74 Δ*phoA532*	$MG1655_{MC}$ via BW13183	Pro$^+$ with P1 on BW13034
BW13635	*proC*::Tn*5-132*	BD792	Tc^r with P1 on BW10244
BW13719	DE3(*lac*)X74 *mel*::Tn*5*seq1 *phn*(EcoB) Δ*phoA532*	$MG1655_{MC}$ via BW12720	Kan^r Mel$^-$ with λ::Tn*5*seq1
BW13745	DE3(*lac*)X74 Δ*phoA532 phn*(EcoK^0)	BD792 via BW13635	Pro$^+$ with P1 on BW13247
BW13908[c]	DE3(*lac*)X74 *phn*(EcoB) Δ(λ *N7 N53 cI857* H1 *chl bio-275*) Δ*phoA532*	$MG1655_{MC}$ via BW13893	Gal$^+$ with P1 on BW13591
BW14001	DE3(*lac*)X74 Δ(*mel proP phnC-phnP*)*2*::Tn*5*seq1/*132*(*tet*) Δ*phoA532*	$MG1655_{MC}$ via BW13719	Tc^r with P1 on BW12466[d]
BW14003	DE3(*lac*)X74 *mel*::Tn*5*seq1 *phnP3*::Tn*5-132 phn*(EcoB) Δ*phoA532*	$MG1655_{MC}$ via BW13719	Tc^r with P1 on BW12478[d]
BW14031	Δ(*putPA*)*101 phn*(EcoK$^+$) *trp thi*	EM41	Spontaneous Pn$^+$
BW14331	DE3(*lac*)X74 Δ*phoA532* Δ(*mel proP phnC-phnP*)*2*::Tn*5*seq1/*132*(*tet*)	BD792 via BW13745	Tc^r with P1 on BW14001[d]
BW14332	DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532*	BD792 via BW14331	Mel$^+$ with P1 on BW10748
BW14439	*lac-169* Δ(*pstCAB phoU bglCSB*)*201* P_i^- Δ(*mel proP phnC-phnP*)*3*::Tn*5*seq1/*132*(*tet*)	BD792 via BW4974	Tc^r with P1 on BW14108[d]
BW14455	*lac-169* Δ(*pstCAB phoU bglCSB*)*201* P_i^- *phn*(EcoB)	BD792 via BW14439	Mel$^+$ with P1 on BW12720
BW14696[b]	DE3(*lac*)X74 *phn*(EcoB) Δ*phoA532* Δ(*phoBR*)*1/527*::Tn*5*(SacII)	BD792 via BW14332	Kan^r with P1 on BW12808
BW16099	DE3(*lac*)X74 *serB*::Tn*5-132 phn*(EcoB) Δ*phoA532*	BD792 via BW14332	Tc^r with P1 on BW10735

[a] All strains are *E. coli* K-12.

[b] Δ(*phoBR*)*1/527*::Tn*5*(SacII) is a Tc^s recombinant of BW12187 which has two Tn*5* insertions, one upstream and another downstream of the *phoBR* operon. Tn*5*(SacII) has a deletion of the *Sac*II fragment between sites in the Tn*5* central region and IS*50*R (unpublished data).

[c] See Materials and Methods.

[d] See Table 2.

tive control strains were always compared on the same plate. The X-P_i character was tested at 30°C because the blue color due to the *phn*(EcoB) allele was darker at lower temperatures. The *fdhF* phenotype was tested by the agar overlay technique (40) with both M9s and BW13908 as formate dehydrogenase-negative controls; the *proP* dehydroproline resistance phenotype was scored by the radial streak test (55) after each mutation was crossed into the *putPA* mutant EM41 with P1. The melibiose phenotype was scored on melibiose-MacConkey agar at 30°C.

BW4794 was used to test the P_i character of *phn* alleles. BW4794 has a P_i-negative (P_i^-) defect, the phenotype of which requires two lesions in the *ilv* and *pstSCAB-phoU* region: Δ(*pst bgl*)*201* and an undefined but linked mutation. [Δ(*pst bgl*)*201* leaves *pstS* intact but removes much of the DNA near and beyond restriction sites in the *pstA* gene.)

TABLE 2. Mapping strains

Allele	*phn*::Tn*5-132* mutant[a]	BW12720 transductant[b]	Tn*5-112* swap[c]	Tn*5-235* swap[d]	Δ(*mel phn*)::Tn*5*seq1/*132* (*tet*) mutant[e]	Δ(*mel phn*) Δ(*putPA*) transductant[f]
phnE1	BW12465	BW12917	BW13196	BW14251	BW13885	BW14149
phnH4	BW12479	BW12922	BW13201	BW14254	BW14006	BW14075
phnJ7	BW12556	BW12925	BW13205	BW14257	BW14012	BW14076
phnK6	BW12555	BW12924	BW13204	BW14256	BW14009	BW14151
phnO5	BW12554	BW12923	BW13202(−) and BW13203(+)	BW14255	BW13888	BW14150
phnP2	BW12466	BW12919	BW13197	BW14252	BW14001	BW14077
phnP3	BW12478	BW12920	BW13199	BW14253	BW14108[g]	BW14152

[a] Mutants were made in BW10748.

[b] Tc^r transductants of BW12720 were made with P1 on the mutants in column 2.

[c] Kan^r Tc^s swaps of the transductants in column 3 were made with λ::Tn*5-112*. BW13202(−) and BW13203 (+) are Pn$^-$ and Pn$^+$, respectively, and therefore have Tn*5-112* in opposite orientations. See the text for details.

[d] Kan^r Tc^s swaps of the transductants in column 3 were made with λ::Tn*5-235* (Lac$^+$).

[e] Each mutant, except for BW14108, was made in P1 crosses with the *phn*::Tn*5-132* mutant in column 2 as the donor and BW13719 (*mel*::Tn*5*seq1) as the recipient. Deletion recombinants were identified as Tc^r Kan^s Mel$^-$ Pn$^-$ recombinants as described in the text.

[f] Tc^r transductants of Δ(*putPA*) mutants were made with P1 on the Δ(*mel phn*)::Tn*5*seq1/*132*(*tet*) mutants in column 6. EM41 is the parent of BW14075 to BW14077, and BW14031 is the parent of BW14149 to BW14151.

[g] BW14108 is a Tc^r Kan^s swap of BW14107 [Δ(*mel proP phnC-phnP*)*3*::Tn*5*seq1/*132*(*kan*)] which was made with λ::Tn*5-132*. BW14107 is a Tc^s recombinant of BW14003 (*mel*::Tn*5*seq1 *phnP3*::Tn*5-132*) (Table 1). See the text for details.

BW4794 was made in a P1 cross in which both parents were phenotypically P_i positive (P_i^+), so the source of the P_i^- lesion was uncertain. To make BW4794, we selected Ilv$^+$ transductants on glucose-M63 agar (49) and tested them for their Bap and P_i phenotypes. About 90% of the Bap constitutive transductants were P_i^+; the remainder, like BW4794, were, unexpectedly, P_i^-. BW4794 grows well on glucose-M63 agar, which contains 100 mM P_i, and on glucose-MOPS agar with 2 mM α-glycerol phosphate; it grows poorly on media with 2.0 mM P_i and does not grow on media with 0.1 mM P_i (unpublished data).

Transposon mutagenesis and recombinational switching of Tn*5* elements. BW10748 [*phn*(EcoB)] was infected with λ::Tn*5-132*, and tetracycline-resistant (Tc^r) mutants, selected on tryptone-yeast extract agar with tetracycline were replica plated onto glucose-MOPS-MPn and 2 mM P_i agars to identify Pn-negative (Pn$^-$) mutants and simple auxotrophs. BW13719 is a Kan^r mutant of BW12720 made with Tn*5*seq1; BW13719 was selected on tryptone-yeast extracts-kanamycin agar and was Mel$^-$ (white) when replica plated onto melibiose-MacConkey agar.

Tn*5* elements with different central regions (Tn*5-112*, Tn*5-132*, Tn*5-235*, and Tn*5*seq1) were switched one for another to provide markers in strain constructions, to test for polarity, to map mutations, and to make deletions between two Tn*5* insertions or between Tn*5-132* and Mu d1-1734. Such recombination events occur via IS*50* homologies in the Tn*5* element(s) and Mu d1-1734 (Fig. 1). Chromosomal Tn*5* insertions with one drug marker were exchanged in transductional crosses with a λ::Tn*5* element with a different drug marker. Recombinants were identified as transductants that lost the resident drug marker; e.g., *phn*::Tn*5-132*(*tet*) insertions were switched with λ::Tn*5-112*(*kan*) or Tn*5-235*(*kan*) and the recombinants were identified as Kan^r Tc^s transductants.

Nomenclature of Tn*5*- and Mu d1-generated deletions. Tn*5*seq1 has a deletion of much of IS*50*L but has an intact IS*50*R, except for its outside end (36). As a consequence, two different structures can be formed in recombination between Tn*5*seq1 and Tn*5-132*, depending on the relative orientations of their respective IS*50*s. No attempt was made to determine which structure was formed. Instead, a slash indicates a hybrid element in which one end is from Tn*5*seq1 and the other is from Tn*5-132*, e.g., Δ(*mel proP phnCDE*)*1*::Tn*5*seq1/*132*. Tn*5*seq1/*132*(*tet*) and Tn*5*seq1/*132*(*kan*) refer to hybrid elements with the same outside ends and with central regions for Tc^r and Kan^r, respectively. According to our nomenclature scheme, Δ(*mel proP phnD*)*31*::Tn*5*seq1/1734 was made by a recombination event between homologous IS*50*s in Tn*5*seq1 and Mu d1-1734. Numerals following the parentheses signify the allele of the transposon insertion(s) used to create the respective deletion.

Selection of revertants. Pn$^+$ Pt-positive (Pt$^+$), or phosphoserine-positive revertants were selected from 1-ml stationary-phase LB cultures as follows. Cells were collected by centrifugation, resuspended in 0.85% NaCl (without washing), and poured onto glucose-MOPS agar, and the excess liquid was evaporated in a sterile 37°C environment. Colonies that arose in less than 7 days were purified nonselectively and tested. Most cultures produced only a few mutants, except as noted in the text.

RESULTS

New P assimilatory phenotypes associated with the *phn* gene cluster. We tested *E. coli* K-12, which has the cryptic *phn*(EcoK^0) allele (53), for the use of reduced P as a sole P source and discovered that it mutates to use Pt in the same way as it mutates to use Pn. Importantly, mutants selected for the use of Pt or Pn are able to use either P compound, showing that the metabolism of Pt and the metabolism of Pn are related (Table 3, line 1). We also tested *E. coli* K-12 for the use of various P_i esters. As expected, because Bap is a periplasmic nonspecific phosphomonoesterase, *phoA*$^+$ strains use P_i esters thought not to be transported into the cytoplasm. However, while *phoA* mutations abolish the use of phosphoserine or X-P_i as a sole P source, *phoA* mutants yield pseudorevertants which have second-site mutations and which are P_i ester positive. Such pseudorevertants also arise by activation of the *phn*(EcoK^0) allele because the mutations are linked to the *phn* gene cluster (data not shown). Furthermore, *phoA* mutants mutate to use Pn, Pt, and P_i esters simultaneously, regardless of which P source is used in the selection (Table 3, lines 1, 2, and 3). All such mutants therefore have a mutationally activated *phn* (EcoK$^+$) allele. This fact is further supported by the finding

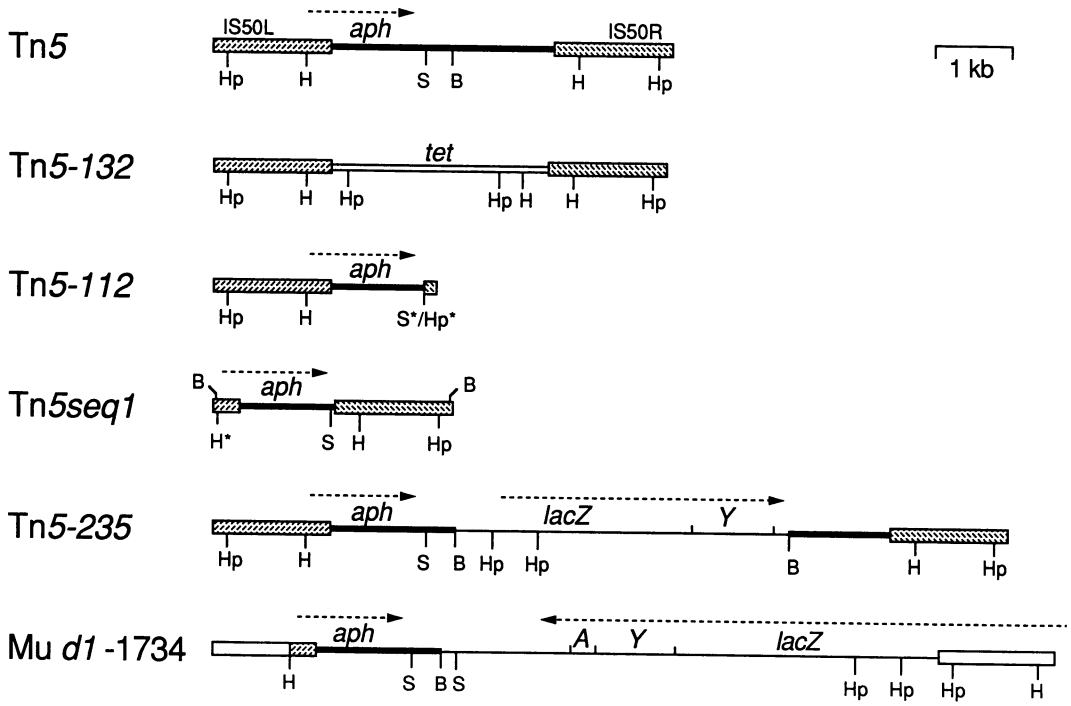

FIG. 1. Physical structures of Tn*5* elements and Mu d1-1734. Tn*5*seq1 and Tn*5-235* are Kan^r and transposition proficient; Tn*5-112* is Kan^r and transposition deficient; and Tn*5-132* is Tc^r and transposition proficient (7). Stippled boxes show IS*50* DNAs in common in the Tn*5* elements and Mu d1-1734 (12). Broken arrows show directions of transcription for the *aph* (*kan*), *lacZY*, and *lacZYA* genes. B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sal*I. Sites marked with an asterisk were destroyed during construction of the Tn*5* elements.

that *phoA* mutants with the Pn$^+$ *phn*(EcoB) allele can use Pn, Pt, and P_i esters, while *phoA* Δ*phn* mutants cannot; the latter also do not yield mutants (lines 4 and 5). Therefore, the *phn* gene cluster has roles in the use of Pn, Pt, and P_i esters. Since a *phoB* mutant fails to use such alternative P sources (Table 3, line 6), the *phn* gene(s) for Pn, Pt, and *phoA*-independent P_i ester utilization is a member of the PHO regulon. (PhoB is a required transcriptional activator for genes in the PHO regulon [30, 52].)

Transport role for the *phn* gene cluster. On the basis of a DNA sequence analysis, the *phn* gene cluster is thought to encode systems for both Pn uptake and biodegradation (13).

TABLE 3. Role of the *phn* gene cluster in P assimilation

Genotype[a]	Growth on the following P source[b]:					
	AEPn	EPn	MPn	Pt	Phosphoserine	X-P_i
phn(EcoK^0)	*	*	*	*	+	+
*phn*EcoK^0 *phoA*	*	*	*	*	*	*
phn(EcoK$^+$) *phoA*	+	+	+	+	+	+
phn(EcoB) *phoA*	+	+	+	+	+	+
Δ*phn phoA*	−	−	−	−	−	−
phn(EcoB) *phoA phoB*	−	−	−	−	−	−
phn(EcoK^0) *phoA serB*	*	*	*	*	−	*
phn(EcoK$^+$) *phoA serB*	+	+	+	+	w	+
phn(EcoB) *phoA serB*	+	+	+	+	w	+

[a] Strains were BW11331, BW12069, *phn*(EcoK$^+$) mutants of BW12069, BW12720, BW12655, BW14696, BW12348, BW12350, and BW16099, the genotypes of which are given in Table 1.

[b] Tests for growth were done on glucose-MOPS agar with 0.1 mM concentrations of the P sources. *serB* mutants were supplemented with L-serine. Symbols: *, cryptic character which is mutatable to a positive phenotype; +, substantial growth; −, no growth (above the background level seen without any added P source); w, weak growth, clearly an intermediate phenotype.

We considered that the Pn uptake system may nonspecifically transport P_i esters, thus explaining the ability of Pn$^+$ *phoA* mutants to use phosphoserine and X-P_i as sole P sources. To test this idea, we examined the effect of a *serB* lesion. Since the *serB* product, phosphoserine phosphatase, is a cytoplasmic enzyme for the last step in de novo L-serine biosynthesis (22), an effect of a *serB* mutation on the use of phosphoserine is evidence for phosphoserine entry into the cytoplasm. Both *serB phoA phn*(EcoK$^+$) and *serB phoA phn*(EcoB) mutants showed markedly reduced growth on phosphoserine (Table 3, lines 8 and 9). Also, *serB* mutants failed to yield P_i ester-positive mutants on phosphoserine agar (line 7). Some residual growth on phosphoserine was seen in Pn$^+$ *serB* mutants, however. Such growth was likely due to other cytoplasmic phosphatases, since growth on X-P_i was unaffected by a *serB* mutation. Therefore, the *phn* gene cluster probably encodes a transporter for the P_i esters phosphoserine and X-P_i, while growth on these esters is also dependent on one or more cytoplasmic phosphatases.

Further evidence that the *phn* gene cluster encodes a transporter was obtained with BW4794 which, although it has an undefined lesion, behaves as a P_i transport mutant (see Materials and Methods). BW4794 has the cryptic *phn*(EcoK^0) allele and therefore yields Pn$^+$ mutants. All mutants selected on Pn agar also grow on 0.1 mM P_i agar. However, only a portion of BW4794 mutants selected on P_i grow on Pn agar. Apparently, there are alternative mechanisms by which BW4794 can become P_i^+: one mechanism involves the activation of the *phn*(EcoK^0) allele, and the other does not. In addition, BW14455, a *phn*(EcoB) transductant of BW4794, grows well on both Pn and P_i agars. Thus, the *phn*-dependent growth of *phn*(EcoB) strains on Pn, of *phoA* mutants on P_i esters, and of the Δ*pst* mutant

BW4794 on P_i implies that the *phn* gene cluster encodes a transporter(s) for Pn, P_i esters, and P_i.

Isolation of *phn*::Tn*5-132* mutants. We screened approximately 18,000 independent transposon-induced mutants by replica plating for ones that failed to grow on MPn agar. We found seven Pn^- mutants and 47 auxotrophs. Each Pn^- mutant was complemented by the Pn^+ plasmid pBW120 (53), which carries a 15.6-kb *phn*(EcoB) DNA insert. Also, each was linked to the *phn* gene cluster in P1 crosses. We therefore named the alleles *phn-1* to *phn-7* and crossed each into BW12720 [*phn*(EcoB) *phoA*] for further study. Our data are consistent with the following assignments: *phnE1*, *phnH4*, *phnJ7*, *phnK6*, *phnO5*, *phnP2*, and *phnP3* (this paper; unpublished data). These gene assignments are used below.

Each *phn*::Tn*5-132* mutation abolished the use of AEPn, EPn, MPn, PnAc, and Pt on glucose-MOPS agar. To be sure that the growth phenotype was not due to contaminating P_i, we measured the growth yield of the Pn^+ parent in glucose-MOPS broth cultures. Pn^+ *E. coli* gave growth yields per milligram of P on each Pn, except for PnAc, equivalent to those on P_i as a sole P source. The parental strain failed to grow in PnAc broth cultures; it also grew less well on PnAc agar than on other Pn agars. The *phnE1*::Tn*5-132* mutation, unlike the others, abolished the use of P_i esters, in addition to Pn and Pt. Furthermore, the *phnE1* lesion was the only one that abolished growth on P_i agar when crossed into the P_i^- Δ*pst* mutant BW4974. Therefore, PhnH, PhnJ, PhnK, PhnO, and PhnP may have roles in common for the use of Pn and Pt, while PhnE may have a role(s) in common for the use of Pn, Pt, P_i, and P_i esters. All three *phnD*::*lacZ*(Mu d1) insertions described previously (53) also abolished the use of Pn and Pt as well as P_i esters in a *phoA* host (data not shown).

Mapping of *phn*::Tn*5-132* mutations. Genetic linkage to the *phn* gene cluster was tested by crossing each *phn*::Tn*5-132* mutation into three *phnD*::*lacZ*(Mu d1-1734) mutants which are *phoU* and therefore express the *lacZ* transcriptional fusion constitutively. Although all seven proved to be linked, there were significant differences, 98.4 to 54.6%, in the extent of linkage (data not shown). The approximate distance between the most distant lesions, *phnD41* and *phnP2*, is greater than 10 kb, according to the formula of Wu (56). Because the *phn*::Tn*5-132* insertions are mutations of the *phn*(EcoB) allele and the *phn*::Mu d1-1734 insertions are mutations of the *phn*($EcoK^0$) allele, the genetic distances may be overestimates if sequence differences between the *phn*(EcoB) and *phn*(EcoK) alleles are great. Subsequent studies based on physical mapping proved that the distance between the *phnD41* and *phnP2* lesions is about 9.5 kb, however (data not shown).

Polarity in the *phn* gene cluster. Since the *phn* gene cluster likely encodes an operon (13) and Mu d1 and Tn*5-132* insertions are usually polar, the effects of these insertion mutations may be due to inactivation of particular *phn* genes or to polarity or both. In the case of the Tn*5-132* insertions, we were able to determine that the effect of most *phn*::Tn*5-132* mutations was not solely due to polarity by switching each *phn*::Tn*5-132* allele to a *phn*::Tn*5-112* allele. Switching Tn*5-112* and Tn*5-132* occurs via homologous recombination between their outside ends, which are identical (Fig. 1), and leaves Tn*5-112* at the site of the original *phn*::Tn*5-132* but in either of two possible orientations. In one orientation, Tn*5-112*, like Tn*5-132*, is polar; in the opposite orientation, Tn*5-112* is (usually) nonpolar (6). Therefore, switching each *phn*::Tn*5-132* allele to the respective *phn*::Tn*5-112* allele is expected to produce both polar and nonpolar recombinants, in approximately equal proportions. If polarity is the sole basis for the phenotype, about one-half of the recombinants may lose the mutant phenotype. Accordingly, each *phn*::Tn*5-112* was switched for each *phn*::Tn*5-132* allele as described in Materials and Methods. The effects of six of the seven *phn*::Tn*5-132* insertions were not solely due to polarity, because no recombinants of six insertions restored any *phn*-associated phenotype. Thus, the phenotype of these insertions probably reflects the loss of function of the interrupted genes. In contrast, the Pn^- phenotype of the *phnO*::Tn*5-132* insertion was due to polarity, because 38% of the Tn*5-112* recombinants of this insertion had the ability to use Pn and Pt as sole P sources simultaneously restored to them (Fig. 2). Hence, the *phnO* gene has no obligatory role in Pn or Pt utilization.

Reversion analysis. The *phnD*::*lacZ*(Mu d1) mutants were isolated in a *phn*($EcoK^0$) strain; none gives rise to Pn^+ mutants. Their failure to give rise to Pn^+ mutants may be due to the need for two mutational events, one of which, precise excision of Mu, is rare. If precise excision were to occur, a second event would be needed to activate the cryptic *phn*($EcoK^0$) allele. However, the *phn*::Tn*5* insertions described above were isolated in a *phn*(EcoB) *E. coli* K-12 host. Therefore, each is expected to revert in one step via precise excision.

As a means to test further the role of individual *phn* genes in P assimilation, we selected revertants of each *phn*::Tn*5-132* or *phn*::Tn*5-112* mutant for growth on Pn and Pt and of the *phnE1*::Tn*5* mutant for growth on Pn, Pt, and phosphoserine. We selected revertants for three reasons. First, proving that precise excision events are common among phenotypic revertants provides strong evidence that a particular gene is needed. Second, finding revertants not due to precise excision events provides evidence that the gene can be bypassed for a particular function or that it is needed only for a subset of *phn*-associated functions. Third, finding revertants unlinked to the *phn* gene cluster may implicate other genes in Pn metabolism. Both kinds of *phn*::Tn*5* insertions were tested for reversion to circumvent problems due to polarity. In this regard, revertants of the *phn*::Tn*5-112* insertions are expected regardless of whether the parental Tn*5-112* insertion is in the polar or the nonpolar orientation, because recombinational switching in the IS*50*s of Tn*5-112* occurs frequently and can convert a polar Tn*5-112* to a nonpolar Tn*5-112* (6).

Several hundred independent revertants were selected for growth on Pn, Pt, or P_i ester, as appropriate. Each revertant was tested for the loss of the respective Tn*5* drug resistance to distinguish ones due to precise excision events from ones in which function was restored without the loss of Tn*5-132* or Tn*5-112*. All revertants of the *phnE1*, *phnH4*, *phnK6*, *phnP2*, and *phnP3* mutants with either Tn*5-132* or Tn*5-112* insertions were due to precise excision events regardless of whether selection was done on Pn or Pt agar. Revertants of the *phnE1* mutant also were due to precise excision events when growth on phosphoserine was used for selection. Most revertants of the *phnJ7*::Tn*5-132* and *phnO5*::Tn*5-132* mutants also arose by precise excision, although a few did not. Five of the Pn^+ precise excision events (i.e., somewhat <2%) were accompanied by a Tn*5* transposition event, because each revertant acquired a drug resistance marker unlinked to the *phn* locus. Two other exceptional revertants are described below. As expected, all revertants due to precise excision grew on each of the other P compounds, regardless of which one was used to select mutants. Also, as

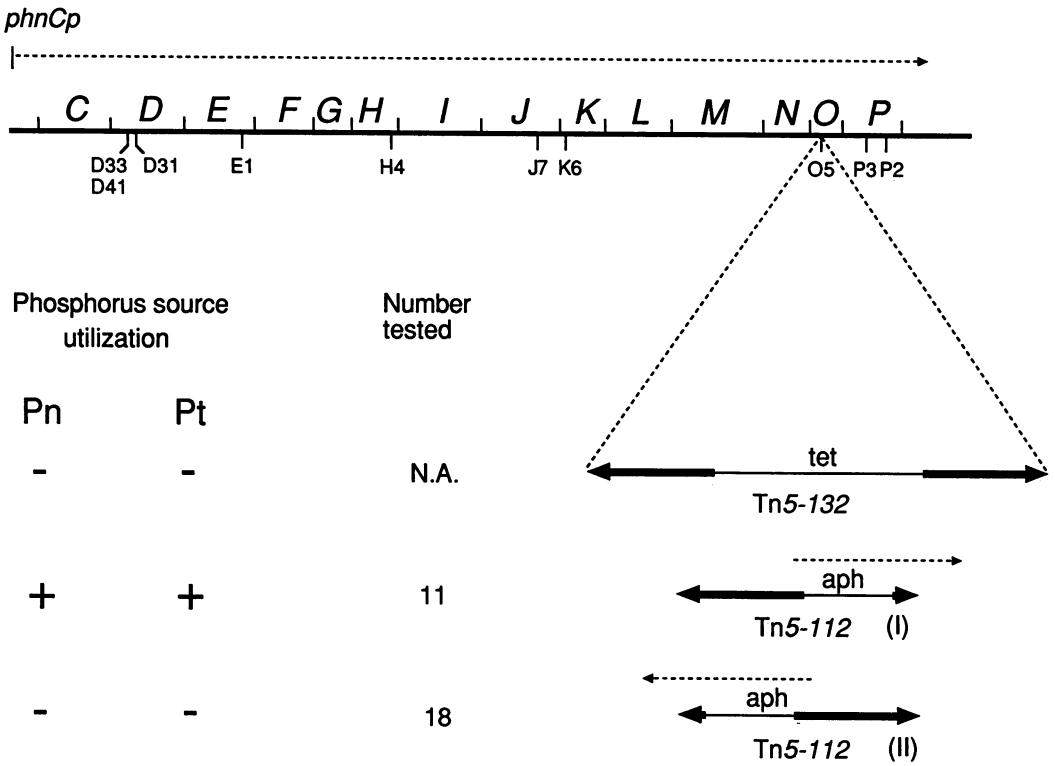

FIG. 2. Polarity in the *phn* gene cluster. The sites of the *phnD31*::*lacZ*(Mu d1) *phnD33*::*lacZ*(Mu d1), and *phnD41*::*lacZ*(Mu d1) insertions were determined by DNA sequence analysis (34). The approximate positions of the *phnE1*::Tn*5*, *phnH4*::Tn*5*, *phnJ7*::Tn*5*, *phnK6*::Tn*5*, *phnO5*::Tn*5*, *phnP3*::Tn*5*, and *phnP2*::Tn*5* insertions are indicated. Upon switching *phnO5*::Tn*5-132* to *phnO5*::Tn*5-112*, 11 of 29 Kan^r Tc^S recombinants became Pn$^+$ and Pt$^+$; the remainder were Kan^r Tc^S Pn$^-$ Pt$^-$. Two recombinant classes are due to oppositely oriented Tn*5-112* insertions (6). In orientation I, downstream DNA is transcribed from the *aph* (*kan*) promoter, because the *112* deletion in IS*50*R of Tn*5-112* removes a terminator. In orientation II, the *aph* promoter faces the opposite direction and the downstream DNA is not expressed. See the text for details. N.A., Not applicable.

expected, these revertants arose infrequently. In contrast, the polar Pn$^-$ *phnO5*::Tn*5-112* mutant BW13202 (Table 2; type II in Fig. 2) yielded frequent pseudorevertants which retained Tn*5-112*. The latter are therefore probably due to recombinational switching of Tn*5-112*.

The *phnJ7*::Tn*5-112* mutant yielded two exceptional revertants on Pt agar which were due to second-site mutations. The same revertants were also exceptional because they were restored for growth on Pt agar but not on Pn agar. In both cases, the presence of the original mutation was verified by crossing it into a new strain with P1. However, both Pt$^+$ Pn$^-$ pseudorevertants of the *phnJ7*::Tn*5-112* mutant still required other *phn* genes to grow, because Δ*phn* derivatives of them were Pt negative (Pt$^-$).

As the vast majority of Pn$^+$ and Pt$^+$ revertants were due to precise excision, our data indicate that the *phnE*, *phnH*, *phnJ*, *phnK*, and *phnP* genes play important roles in both Pn metabolism and Pt metabolism. The finding of unlinked mutations that suppress the *phnJ7*::Tn*5-112* lesion shows that the *phnJ* gene need not be intact for growth on Pt.

Use of Tn*5* elements to construct Δ(*mel proP phn*) deletions. Deletions of the *phn* genes were made for two reasons: first, to aid in defining the *phn*::Tn*5* map locations, and second, to test for functions of intervening *phn* genes. Deletions that remove DNA between Tn*5* insertions in the *mel* and *phn* genes were made in two ways. One method required making double mutants with two Tn*5* elements on the chromosome: one, Tn*5*seq1, was marked with Kan^r, and the other, Tn*5-132*, was marked with Tc^r. Such mutations can give rise to deletions by homologous recombination in the IS*50* DNAs, provided that no essential gene is in the intervening region (Fig. 3A). Deletions were selected as Tc^s recombinants. A second, more convenient, method involved testing P1 transductants for those that acquired deletions in the P1 cross. Many deletions were found in the P1 crosses used to make the double mutants described above. Deletions can be formed in a P1 cross if one recombination event occurs between homologous chromosomal sequences and the other event occurs between the IS*50*s (Fig. 3B). Such P1 transductants have deletions of DNA between the Tn*5* insertion sites in the donor and recipient DNAs. Deletion recombinants were recognized in P1 crosses between the *phn*::Tn*5-132* donors and the *mel*::Tn*5*seq1 recipient as Tc^r transductants that were Pn$^-$, kanamycin sensitive (Kan^s), and Mel$^-$.

In each case, genetic tests proved that the suspected deletions removed DNA between the respective Tn*5* elements. In the case of the Δ(*mel phn*)::Tn*5*seq1/*132* recombinants, both Mel$^-$ and Pn$^-$ were 100% linked to the Tn*5*-encoded drug resistance marker in P1 crosses. (P1 crosses with donor strains with no deletions showed less than 20% linkage between the same markers.) Further evidence that the Δ(*mel phn*)::Tn*5*seq1/*132* mutants were true deletions was provided by showing that each was also ProP$^-$. Thus, the *proP* gene resides in the *mel-phn* interval. All deletion mutants were FdhF$^+$, so the *fdhF* gene is outside this interval. Interestingly, all Δ(*mel proP phn*)::Tn*5*seq1/*132* recombinants were also P$_i$ ester negative, including ones derived from the P$_i$ ester-positive *phnH4*::Tn*5-132*, *phnJ7*::

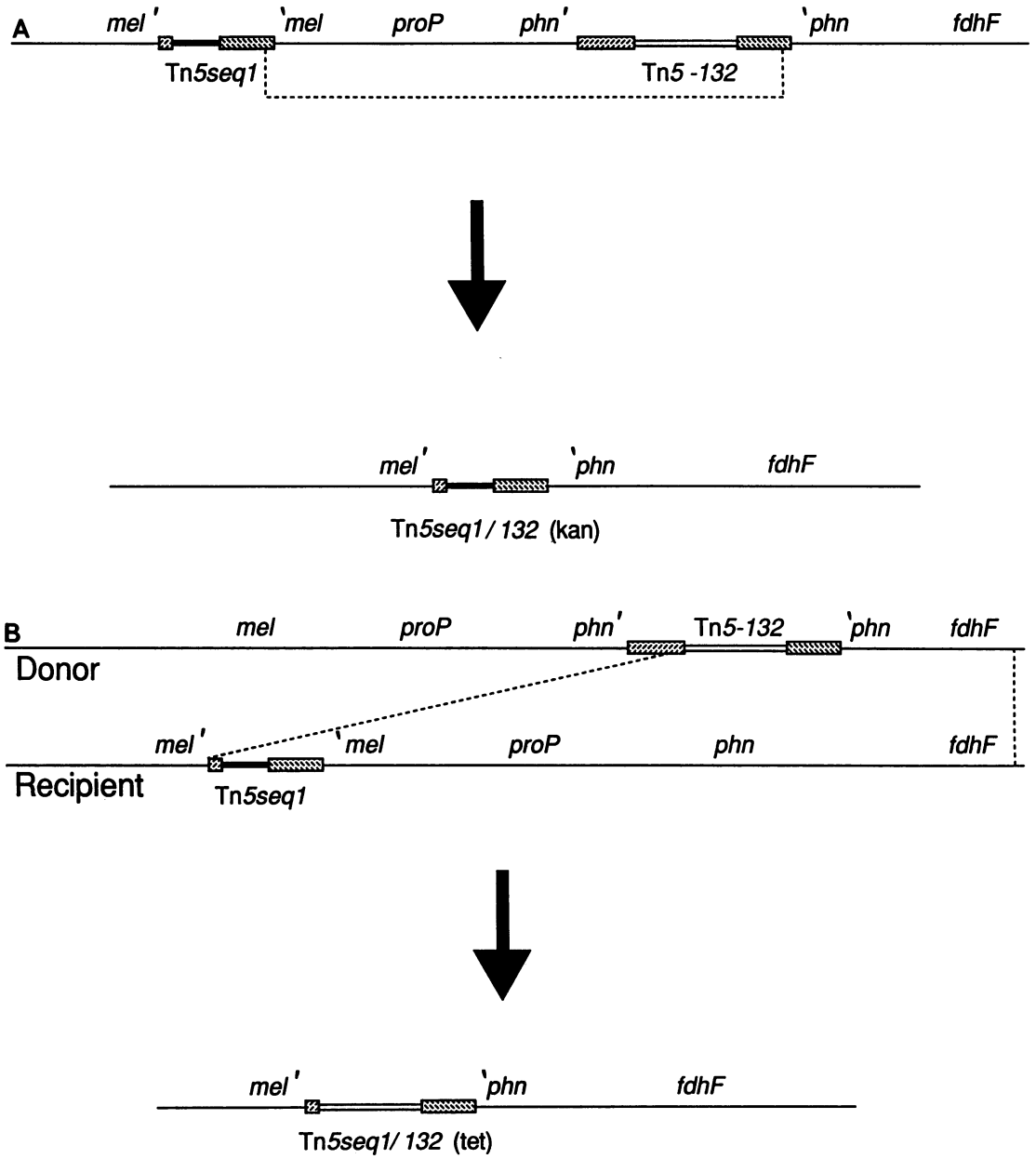

FIG. 3. Use of Tn*5* elements to construct deletions in the *phn* gene cluster. (A) Deletions formed by homologous recombination in mutants with two chromosomal Tn*5* insertions. The broken line shows the approximate location of a single recombination event, and the bottom line shows a resulting deletion. The left- and right-hand primes mark Tn*5* junctions with the *mel* and *phn* genes, to show which junctions are conserved in the deletions. (B) Deletions formed in P1 crosses in which one recombination event occurs between donor and recipient IS*50*s and the second recombination event occurs between homologous chromosomal sequences. The second event may occur anywhere to the right of *phn*::Tn*5*; the panel arbitrarily shows this event on the right side of the *fdhF* gene. The broken lines show recombination events between IS*50* and chromosomal sequences. The primes mark Tn*5* junctions as in panel A. This figure is not to scale.

Tn*5-132*, *phnK6*::Tn*5-132*, *phnP3*::Tn*5-132*, and *phnP2*::Tn*5-132* insertions. Therefore, the Δ(*mel proP phnC-phnH*), Δ(*mel proP phnC-phnJ*), Δ(*mel proP phnC-phnK*), Δ(*mel proP phnC-phnO*), and Δ(*mel proP phnC-phnP*) deletions remove the *phnE* (or other) gene required for the use of P_i esters. The *phnE* gene is expected to be lost in the Δ(*mel proP phn*) mutants if the *phn* gene cluster is transcribed in a counterclockwise direction away from the *mel* operon, as determined below.

Determining the relative order of the *phn*::Tn*5* insertions. Data on two-factor crosses between each *phn*::Tn*5-132* insertion and each *phnD*::Mu d1 insertion are described above. As a means of establishing the relative gene order in a less ambiguous way, we tested the *phn*::Tn*5* insertions in deletion mapping experiments. These experiments involved doing a series of P1 crosses between two sets of strains. The set used as P1 donors contained the Δ(*mel proP phn*)::Tn*5*-seq1/*132*(*tet*) deletions described above. The set used as recipients had a *phn*::Tn*5-235* insertion, which contains a centrally located and constitutively expressed *lac* operon, in place of the original *phn*::Tn*5-132* insertion (Fig. 1). In some crosses, a *phnD31*::*lacZ*(Mu d1-1734) *phoU* mutant, which

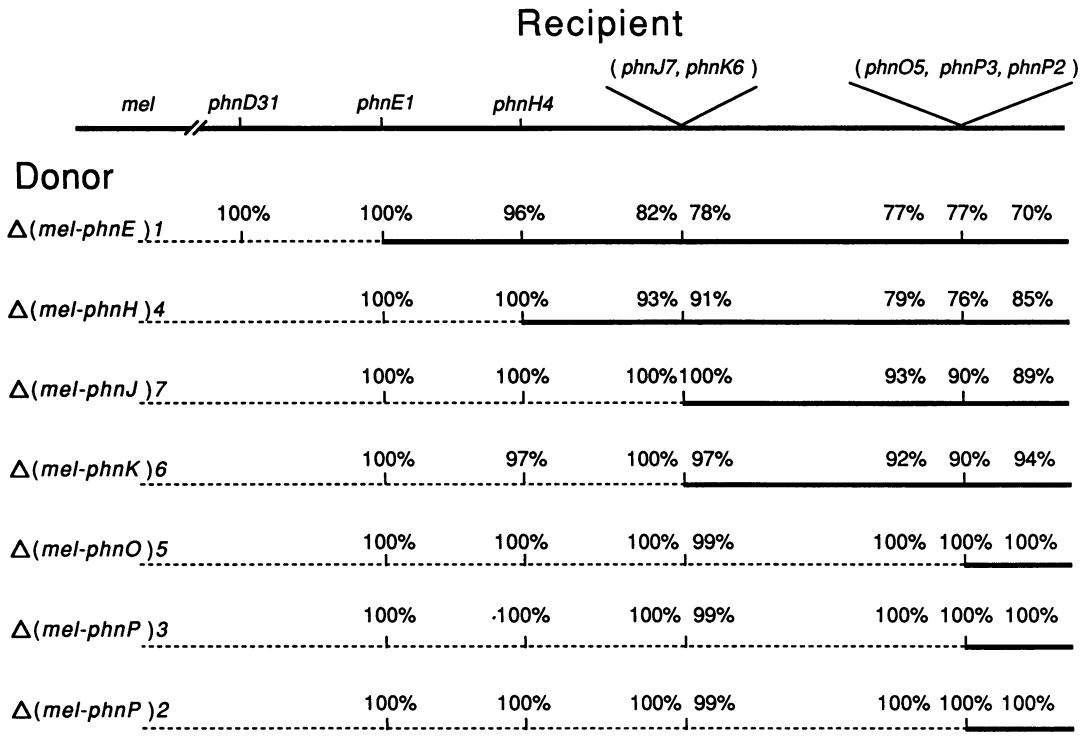

FIG. 4. Use of Δ(*mel proP phn*) deletions to map *phn*::Tn*5* insertions. P1 crosses were done between Δ(*mel proP phn*)::Tn*5-132* donors and Lac$^+$ recipients with *phn*::*lacZ*(Mu d1) or *phn*::Tn*5-235* (Lac$^+$). Several hundred transductants were scored in each cross; the percentages of Lac$^-$ transductants are shown. Some aberrant Lac$^+$ transductants were due to rare transposition events; all transductants were tallied, since probable aberrant events were not verified. See the text for details.

also shows a constitutive Lac$^+$ phenotype, was used as a recipient. The use of recipients with an expressed *lacZ* gene in different *phn* genes allowed us to score large numbers of transductants on the basis of their Lac phenotypes to detect those resulting from recombinational events within the *phn* gene cluster. Accordingly, P1 donors produce only Lac$^-$ recombinants if the deletion in the donor removes the site of the recipient *phn*::Tn*5-235*. Conversely, P1 donors produce Lac$^+$ recombinants if the donor deletion does not remove the site of the recipient *phn*::Tn*5-235*, in which case the proportion of Lac$^+$ transductants is a measure of the distance between the deletion endpoint and *phn*::Tn*5-235*. The results (Fig. 4) show the relative order based on these crosses. *phnE1* and *phnH4* were genetically separable from each other and from the more downstream lesions. Although the *phnJ7*::Tn*5* and *phnK6*::Tn*5* insertions were separable from the *phnO5*::Tn*5*, *phnP3*::Tn*5*, and *phnP2*::Tn*5* insertions, they were inseparable from each other, as were the *phnO5*::Tn*5*, *phnP3*::Tn*5*, and *phnP2*::Tn*5* insertions. Our inability to separate closely linked Tn*5* insertions was probably due to recombinational events between Tn*5* sequences which were indistinquishable from recombinational events between chromosomal sequences. The relative positions indicated for the *phnJ7*::Tn*5* and *phnK6*::Tn*5* insertions and the *phnO*::Tn*5* and *phnP*::Tn*5* insertions are based on results from two-factor crosses with the three *phnD*::Mu d1 mutants described above and are consistent with the results of physical mapping experiments (unpublished data).

Use of Tn*5* elements to construct deletions within the *phn* gene cluster. On the basis of effects on P_i ester utilization, PhnE is likely a transport protein. Conversely, PhnH, PhnJ, PhnK, PhnO, and PhnP are not. Deletions removing DNA between Tn*5-132* insertions in the *phnH*, *phnJ*, and *phnK* genes and the *phnP2*::Tn*5-112* insertion were made as a means to assess the need for other genes in the *phnH-phnP* interval in P_i ester utilization as follows. Double mutants were made with the *phnP2*::Tn*5-112* insertion and the *phnH4*::*Tn5-132*, *phnJ7*::Tn*5-132*, and *phnK6*::Tn*5-132* insertions. Deletions were selected as Tc^s recombinants. One representative of each type was proven to be deleted in P1 crosses. By using as recipients *phn*::Tn*5-235* insertions and as donors the suspected Δ(*phn*)::Tn*5-132* deletions (which were made by switching Tn*5-132* for Tn*5-112* in each deletion mutant), we found the respective Δ(*phnHIJKLMNOP*)*4*/*2*::Tn*5-132*, Δ(*phnJKLMNOP*)*7*/*2*::Tn*5-132*, and Δ(*phnKLMNOP*)*6*/*2*::Tn*5-132* deletions to be 100% linked with both deletion endpoints. Since each mutant remained P_i ester positive, no gene in the *phnH-phnP* interval is needed for P_i ester use.

Orientation of the *phn* gene cluster. We determined the orientation of the *phnD*::*lacZ*(Mu d1-1734) insertions on the chromosome as a way to define the direction of transcription. Since we ascertained the relative locations of the *mel* operon, the *proP* gene, and the *phnD*::Mu d1-1734 and *phn*::Tn*5-132* insertions above, our experiments involved simply showing whether the *phnD*::*lacZ* fusions were transcribed toward or away from these markers. To do this, we took advantage of the 300 bp of IS*50* DNA in Mu d1-1734 (Fig. 1) which allows the formation of deletions via homologous recombination between the *phnD*::Mu d1-1734 and *phnD*::Tn*5-132* insertions in the region. Such deletions are expected to be Lac$^-$ if Tn*5-132* is upstream and Lac$^+$ if Tn*5-132* is downstream. In P1 crosses, the *mel*::Tn*5*seq1-*132* donor produced Lac$^-$ recombinants which had deletions,

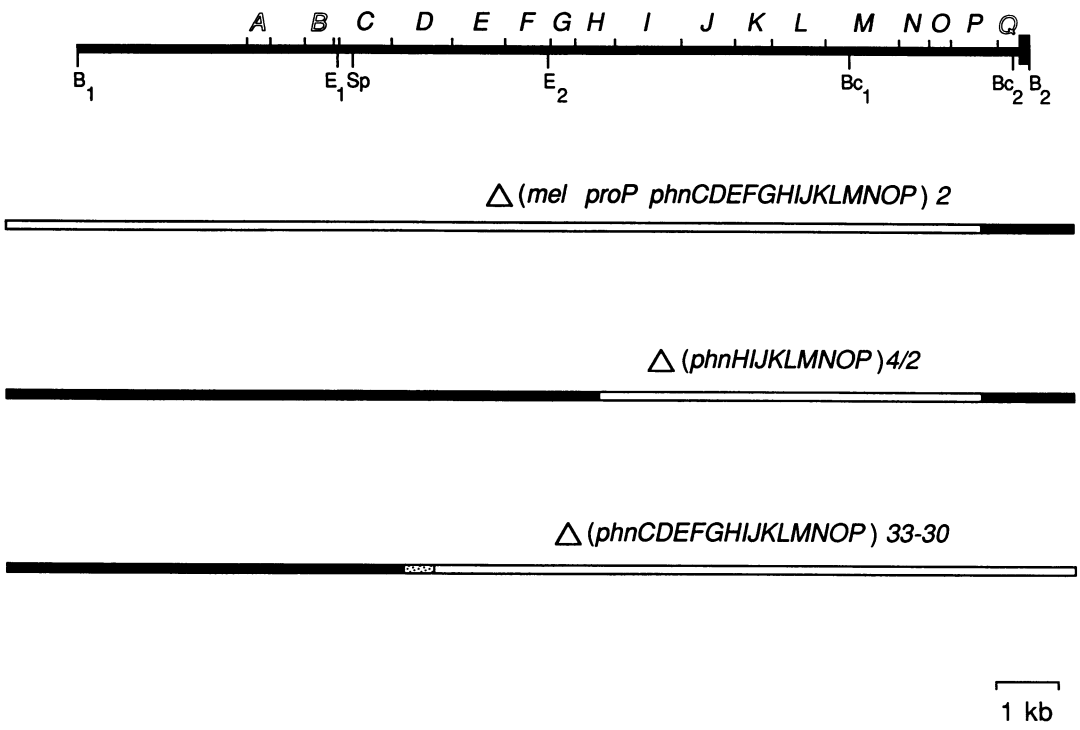

FIG. 5. *phn* gene cluster. The solid bar in the top line shows the 15.6-kbp insert in pBW120, the sequence of which was determined (13). The *Bam*HI (B), *Bcl*I (Bc), *Eco*RI (E), and *Sph*I (Sp) sites are marked. The genes (*phnC* to *phnP*) needed for Pn use are marked in black letters; the *phnA*, *phnB*, and *phnQ* genes, which can be deleted or mutated without an effect on Pn use, are marked in white letters. The open bars indicate deletions. The left endpoint of the Δ(*phn*)*33-30* deletion is marked with a stippled box, because it may lie in the *phnC* or *phnD* gene. The solid bars in the lower three lines show chromosomal DNA which is present. See the text for details.

while the *phnE1*::Tn*5-132* donor produced only Lac^+ recombinants. Therefore, the *phnD* gene is transcribed away from the *mel* gene in a counterclockwise orientation on the chromosome. Furthermore, if the entire *phn* gene cluster is transcribed as a single unit, then transcription is from the *phnC* gene to the *phnP* gene. The same orientation was inferred from the *phn* DNA sequence and a comparison of its restriction map with that of the *E. coli* genome (27, 53).

Complementation tests on *phn* mutants. Plasmid pBW120 has a 15.6-kb insert and was isolated by complementation of the Δ(*phnD-phnP*)*33-30* mutant (Fig. 5). Although Δ(*phn*)*33-30* has one endpoint beyond the *phnP* gene on the right, its other endpoint is within the *phnC* gene or the *phnD* gene near the site of the *phnD33*::Mu d1 (34) from which the deletion was derived, as determined on the basis of blot hybridization of chromosomal DNAs (53). Also, the removal of DNA to the left of the *Sph*I site or to the right of the $BclI_1$ site in pBW120 (Fig. 5) abolished complementation (53). As determined on the basis of the DNA sequence of the insert in pBW120 (13), the *Sph*I site is in the *phnC* gene. Since the *Sph*I-deleted plasmid is both Pn^- and P_i ester negative, the *phnC* gene is needed for the use of Pn and P_i esters.

DNA sequence data revealed 17 open reading frames in a proposed operon which includes the *phnA* to *phnQ* genes (13). In this regard, the *phnA*, *phnB*, and *phnQ* genes were included solely on the basis of sequence information. Importantly, the Δ(*phn*)*33-30* deletion, which was used previously (13), was not useful in tests of whether the *phnA* and *phnB* genes are needed because this deletion does not remove these genes. However, the Δ(*mel proP phnC-phnP*)*2* deletion described above removes all *phn* sequences upstream of *phnP2*::Tn*5*. We therefore subcloned the $EcoRI_1$-$BamHI_2$ fragment into a suitable vector and tested this fragment for complementation of the Δ(*mel proP phnC-phnP*)*2*::Tn*5-132* mutant BW14001 (Fig. 5). Such plasmids fully restored growth on all P sources tested. We conclude that the *phnA* and *phnB* genes have no essential role in Pn or Pt utilization. The *phnQ* gene is also not needed, because a plasmid with an insertionally inactivated *phnQ* gene complemented the Δ(*phn*)*33-30* mutant (data not shown). In addition, we recently identified a promoter for the *phnC* gene in the $EcoRI_1$-*Sph*I interval, the transcription of which is both P_i regulated and PhoB dependent (33a). Therefore, the *phnC* to *phnP* genes constitute the entire *phn* gene cluster, as defined by growth, in appropriate hosts, on Pn, Pt, P_i esters, and P_i as sole P sources.

Characterization of *phn*(EcoK)::Tn*10*. In recent studies on Tn*phoA* (33), Manoil and co-workers (32a) independently isolated the X-P_i-positive mutant LS205 in a *phoA* host. From this mutant they isolated the Tn*10*-induced X-P_i-negative derivative LS206 and showed that both lesions map near 92 min (32a). Since the *phn* gene cluster has a role in P_i ester use, we tested LS205, LS206, and transductants made with these mutants as donors for various *phn*-associated phenotypes. The X-P_i-positive mutant LS205, unlike its parent, was Pn^+. Hence, its X-P_i-positive phenotype is due to a mutationally activated *phn*($EcoK^+$) allele. Furthermore, the X-P_i-negative lesion in LS206 is due to *phn*::Tn*10*, because it abolished growth on Pn agar and was tightly linked to our *phn*::Mu d1 and *phn*::Tn*5* mutations (data not shown). Therefore, we denoted the lesion in LS206 *phn-8*($EcoK^+$)::Tn*10*.

Characterization of *phn*(EcoK)::Tn*5*. While this work was in progress, another laboratory reported the isolation of four

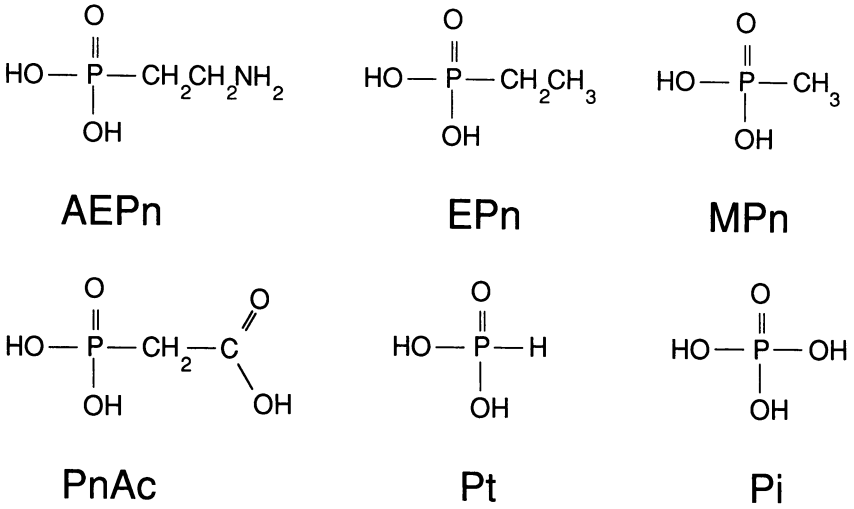

FIG. 6. Structures of AEPn, EPn, MPn, PnAc, Pt, and P_i. The nonionized forms are shown. At a neutral pH, one hydroxyl group on P is ionized in AEPn, EPn, MPn, PnAc, and Pt, while two are ionized in P_i. The amino group of AEPn and the carboxyl group of PnAc are also ionized at a neutral pH. Pt is shown as its more stable tautomer, phosphonic acid. P is in the +5 oxidation state in AEPn, EPn, MPn, and PnAc and in the +3 oxidation state in Pt, since carbon is more electronegative than is hydrogen (14). See the text for details.

Tn*5*-induced Epn-negative mutants (3). However, only one mutant, SL724, proved to be Pn^- and had Tn*5* in the *phn* region (data not shown). The others, SL1158, SL7910, and SL7911, behaved like the parent *E. coli* K-12 strain RB791 and had the cryptic *phn*($EcoK^0$) phenotype. That is, they gave rise to Pn^+ mutants on AEPn, EPn, MPn, and Pt agars. The latter four strains were also *phoA*$^+$ *phoB*$^+$, as they showed normal P_i control of Bap synthesis (data not shown). Since SL1158, SL7910, and SL7911 grew more slowly than did the parent, even on glucose-MOPS-P_i media, they appeared to yield fewer Pn^+ mutants. Apparently, their slower growth led to their mistaken identification as Pn^- mutants.

When the lesion in SL724 was tested for linkage to the *phn* gene cluster, SL724 was shown to contain two Tn*5* insertions, one in the *phn* region and one unlinked (data not shown). We denoted *phn*::Tn*5* in SL724 *phn-9*($EcoK^0$)::Tn*5*. To separate *phn-9*::Tn*5* from the unlinked Tn*5*, SL724 was made Tc^r with λ::Tn*5-132*, the Tc^r recombinants were pooled, and P1 grown on this pool was used to infect BW13745 [*phn*($EcoK^0$) *phoA*]. A total of 87.5% (35 of 40) of the Tc^r transductants were nonmutable on AEPn, EPn, MPn, and Pt agars; all were mutatable on phosphoserine agar. Therefore, *phn-9*::Tn*5* abolishes the use of Pn and Pt but does not affect growth on P_i esters.

We also examined plasmid pSL263, which was isolated on the basis of its ability to complement SL724 for growth on EPn (28). pSL263 failed to complement our Δ(*phnC-phnP*) mutants for growth on AEPn, EPn, or MPn, however. Instead, pSL263 transformants gave rise to Pn^+ mutants. Therefore, Loo et al. (28) probably cloned the cryptic *phn*($EcoK^0$) allele in its inactive form.

DISCUSSION

By testing *E. coli* for the use of various P compounds (Fig. 6) as sole P sources, we discovered three new phenotypes which, in addition to Pn utilization, are associated with the *phn* gene cluster. Also, like the use of Pn, each is cryptic in *E. coli* with the *phn*($EcoK^0$) allele, is expressed without the need for mutational activation in *phn*(EcoB) hosts, and is abolished in *phn*::Mu d1 and *phn*::Tn*5* mutants. Because the *phn* (*psiD*) promoter is induced 100-fold by P_i limitation in a PhoB- and PhoR-dependent manner (53, 54), the *phn* gene cluster probably has a role(s) in P assimilation which, as determined on the basis of the results of this study, is the use of Pn, Pt, P_i esters, and P_i.

Seven *phn*::Tn*5-132* mutants were isolated in a *phn*(EcoB) host. Altogether, eight *phn*::Tn*5* insertions, three *phn*::Mu d1 insertions, and one *phn*::Tn*10* insertion were characterized. Each maps near 92.8 min on the chromosome. However, as determined on the basis of recombination in P1 crosses, the *phn*::Tn*5-132* and *phn*::Mu d1 insertions span more than 10 kbp, indicating that several genes are involved in Pn utilization. In addition, the minimum size of the plasmid DNA insert needed to complement a Δ*phn* host is over 10 kbp (53; this paper). Therefore the complete nucleotide sequence of a 15.6-kbp DNA segment which includes the *phn* gene cluster was determined. The sequence has 17 open reading frames, denoted *phnA* to *phnQ*, in a possible operon arrangement (13). On the basis of the results of this study, we propose that 14 genes, *phnC* to *phnP*, constitute the entire *phn* gene cluster needed for Pn utilization.

The *phnE1*::Tn*5-132*, *phnH4*::Tn*5-132*, *phnJ7*::Tn*5-132*, *phnK6*::Tn*5-132*, *phnO5*::Tn*5-132*, *phnP3*::Tn*5-132*, and *phnP2*::Tn*5-132* mutants failed to use all the Pn tested and Pt. The *phnE1* mutation, in addition, abolished the use of P_i esters in a *phoA* host and the use of P_i in a P_i^- Δ*pst* mutant. To determine which phenotypes were due to inactivation of the particular *phn* gene and which were due to polarity on a downstream gene(s), we converted each *phn*::Tn*5-132* recombinant to a *phn*::Tn*5-112* recombinant. As expected for an effect due to polarity, Pn utilization and Pt utilization were restored in about one-half of the *phnO5*::Tn*5-112* recombinants. No other Tn*5-112* recombinants were either Pn^+ or Pt^+; *phnE1*::Tn*5-112* was not Pi-ester positive. Thus, PhnE, PhnH, PhnJ, PhnK, and PhnP have obligatory roles in Pn metabolism and Pt metabolism, while PhnO has no such role. PhnE also has a role in the use of P_i esters and P_i in *phoA* hosts and P_i^- Δ*pst* hosts, respectively.

We made mutants with deletions between the sites of *phn*::Tn*5* insertions as a means of defining the structure of the *phn* gene cluster and the role of *phn* genes not mutated

with Tn5. All deletions of the *mel-phn* interval are P_i ester negative in *phoA* hosts. This is consistent with the loss of the *phnE* gene in each Δ(*mel phn*) mutant because the *phnE1*::Tn5 mutant is Pi ester negative and the *phnC-phnP* gene cluster is oriented away from the *mel* operon. Because Δ(*mel phn*) mutants are also ProP$^-$ but Fdh$^+$, the gene order of the 92- to 93-min region is clockwise: *fdhF phnPONMLKJIHG FEDC proP mel*. We also used *phn*::Tn5 insertions to make deletions internal to the *phn* gene cluster. Because deletions removing *phnH* and downstream genes are P_i ester positive, no gene in the *phnH-phnP* interval is needed for the use of P_i esters in *phoA* hosts.

Deletions made by recombination between two Tn5 elements or between Tn5 and Mu d1-1734 leave a hybrid element in place of the deleted DNA. Such hybrid elements encode kanamycin or tetracycline resistance (Fig. 3) and are transposition proficient or deficient, depending on how they are made (see Results). The drug resistance markers afforded convenient means for transferring the deletions into new hosts in P1 crosses, e.g., to test the ProP phenotype. In addition, such marked deletions proved useful in mapping mutations in the *phn* gene cluster. Because many removed at least 10 kbp of DNA, the Tn5-generated deletions proved to be especially valuable in characterizing the *phn* gene cluster, which is unusually large.

As a means of determining the orientation of the *phnD*::*lacZ* transcriptional fusion, recombinants between *phnD*::*lacZ*(Mu d1-1734) and *mel*::Tn5-*132* or *phn*::Tn5-*132* insertions were tested for their Lac phenotype. In this way, we proved that the *phn* gene cluster is transcribed in a counterclockwise orientation, i.e., away from the *mel* operon on the *E. coli* chromosome.

Individual *phn* gene products probably have roles in transport, gene regulation, or biochemical catalysis. The phenotypes displayed by the *phn*::Tn5 and Δ*phn* mutants, together with predictions based on the *phn* DNA sequence (13), allow us to suggest some specific roles. Accordingly, we propose that (i) PhnC, PhnD, and PhnE constitute a Pn-specific binding protein-dependent transporter, (ii) PhnO acts as a regulator (at some as-yet-undefined regulatory site), and (iii) other *phn* gene products mostly have roles in a C-P lyase enzyme complex which catalyzes C-P bond cleavage and Pt oxidation and which may be membrane associated.

Binding protein-dependent transporters usually have a periplasmic binding protein and three membrane proteins: a membrane-associated permease with a nucleotide-binding domain and two integral membrane proteins (2). PhnC is a candidate for the permease because PhnC is similar to nucleotide-binding protein members of binding protein-dependent transporters (13). Also, PhnC is needed for growth on P_i esters, because a plasmid with a deletion of the 5′ end of the *phnC* gene has a P_i ester-negative phenotype. Although the negative phenotype may be due to the removal of the *phnC* promoter, it seems unlikely that this removal alone is responsible, because the deletion was made on the high-copy-number plasmid pBW120, a pUC18 derivative (53). PhnD has features in its N terminus in common with those of signal peptides (23), making it a likely candidate for the periplasmic binding-protein component (data not shown). Even though *phnD* mutants are P_i ester negative, no role based on their phenotype can be inferred, because the *phnD*::Mu d1 insertions are polar. PhnE is a candidate for an integral membrane component, because it is similar to other membrane components, in particular, ProW (24; unpublished data). PhnE probably has a transport role, because the *phnE1* mutant is negative for the use of P_i esters and P_i in appropriate hosts. Since the proposed PhnCDE transporter has a single integral membrane component, the Pn transporter may be similar to transporters for arabinose (43), glutamine (37), and ribose (4) which, unlike most binding protein-dependent transporters, have a single integral membrane component. That this transporter is Pn specific is likely, because the *phnCDE* genes are grouped together with nontransport genes needed for Pn (and Pt) utilization. Genes for other transporters, e.g., for ribose (4), exist in operon arrangements with genes having a related metabolic or regulatory function (2). Also, the transport of P_i esters and P_i is probably nonspecific. That *phn*-dependent uptake of P_i esters is nonspecific is favored, because such esters are normally hydrolyzed in *phoA*$^+$ cells by Bap, the synthesis of which is coordinately induced with the *phn* gene cluster. That the transport of P_i is nonspecific is likely, because it would be redundant for the cell to have a second high-affinity P_i transporter together with the PstSCAB transporter, both of which are made during P_i limitation.

The *phn* gene cluster was previously thought to encode an uptake system, because several genes showed sequence similarities to components of binding protein-dependent transporters. In particular, it was noted that PhnC, PhnK, PhnL, and PhnN are each similar to nucleotide-binding protein components, while PhnM is similar to integral membrane components (13). However, of these, only PhnC now appears to have a role in transport, because the Δ(*phnHIJKLMNOP*) deletion has no effect on the use of P_i esters. The *phnF* and *phnG* genes were the only ones not tested for effects on the P_i ester phenotype, because all deletions that removed *phnFG* DNA extended into or beyond the *phnE* gene, which alone causes a P_i ester-negative phenotype. However, no sequence similarities which even remotely suggest a role in transport exist in PhnF or PhnG (data not shown).

Both PhnL and PhnO are candidates for regulatory proteins, because each has a helix-turn-helix DNA-binding motif (13). The finding that a nonpolar *phnO* lesion shows no metabolic defect lends support to a regulatory role for PhnO. Nevertheless, neither PhnL nor PhnO has a role in P_i control of the *phnD*::*lacZ* fusions, because a Δ(*phnC-phnP*) deletion was without effect on the expression of the *phnC* promoter (unpublished data). Since the transcriptional organization of the *phnC-phnP* gene cluster is not understood, PhnO may act elsewhere. With regard to an operon arrangement, only the *phnP* gene has yet been shown to lack its own promoter, on the basis of the polar effect of the *phnO*::Tn5 insertion.

We tested for a role of the *phn* gene cluster in the use of Pt and, in *phoA* hosts, of P_i esters because we found that the use of Pt and P_i esters is a cryptic phenotype, like the use of Pn (53). Because the P_i ester phenotype is cryptic, the difference between the cryptic *phn*(EcoK^0) and Pn$^+$ *phn* (EcoB) alleles must be due to a mutation within *phnCDE*. We previously found an approximate 100-bp difference between *phn*(EcoB) and *phn*(EcoK^0) DNAs, although no differences were found in DNAs of *phn*(EcoK^0) and mutationally activated *phn*(EcoK$^+$) hosts (54). This 100-bp difference may be due to different *phnA-phnB* intergenic regions, where 10 repetitive extragenic palindromic (REP) sequences occur in the *phn*(EcoB) sequence (13). However, this difference cannot account for the different Pn phenotypes, because the extra 100-bp segment in the *phn*(EcoK) DNA is upstream of the *phnC-phnP* gene cluster needed for Pn utilization. Since no gross structural differences in the *phn*(EcoB) and *phn* (EcoK) DNAs exist in the required *phnC-phnP* region, the difference is likely a subtle one. Perhaps a simple null

mutation, such as a base change or a frameshift mutation (53), exists in the *phnC*(EcoK^0), *phnD*(EcoK^0), or *phnE* (EcoK^0) structural gene. In any case, no difference is manifested in P_i control of the *phn*(EcoB) and *phn*(EcoK^0) gene clusters (53).

Gene products not implicated in transport or gene regulation are likely to be involved in biochemical catalysis, although it is difficult to judge what their precise role is or why so many are involved. At least 4 genes (*phnH*, *phnJ*, *phnK*, and *phnP*) and as many as 10 genes (all but *phnC*, *phnD*, *phnE*, and *phnO*) may encode a protein with a metabolic role that cannot be attributed to transport. Accordingly, the *E. coli phnC-phnP* gene cluster may encode a C-P lyase enzyme complex with 4 to 10 protein components. In this regard, another laboratory recently reported cellular C-P lyase activity in an *Enterobacter aerogenes* extract based solely on measurements of P_i production (35). Unfortunately, even though these authors showed that several proteins were needed, no direct comparison between the in vivo C-P lyase in *E. coli* and the reported *E. aerogenes* enzyme can be made without determining the carbon product of the presumed C-P lyase in the *E. aerogenes* extract.

Several reaction schemes for the *E. coli* C-P lyase have been proposed on the basis of in vivo product formation, including ones involving redox chemistry at the P center (15, 44). A biochemical pathway for Pn biodegradation which involves P redox chemistry is particularly attractive, because the *phn*::Tn*5* mutations also abolish growth on Pt. On the basis of the Pt$^-$ phenotype, we propose a two-step process for Pn biodegradation via C-P lyase. Accordingly, reductive cleavage of the C-P bond occurs in one step. This cleavage produces a Pt intermediate, phosphonic acid, in which P is in the +3 oxidation state (Fig. 6). In a subsequent step, C-P lyase oxidizes Pt to form P_i. Interestingly, an NAD-dependent Pt oxidase has been characterized in *Pseudomonas fluorescens* (32). The nucleotide-binding domains in PhnK, PhnL, and PhnN may therefore reflect a role(s) for a dinucleotide(s) needed in the reduction and oxidation steps. The finding of a membrane component (PhnM) suggests that the hypothetical C-P lyase complex may be membrane associated, a characteristic common for enzymes that carry out redox reactions.

Showing that the *phn* gene cluster is needed for the use of reduced P raises new questions about P metabolism, for there is now only scant information on the metabolism of reduced P compounds. A few bacteria, including *E. coli* (10, 31) and some soil bacteria (20, 25), can metabolize P in the +1 or +3 oxidation state. Also, P in the −3 oxidation state, as phosphine (PH_3), is an apparent gaseous product of mixed microbial cultures (17). Our finding in *E. coli* of genes which are necessary for the use of Pt and which are regulated by P_i as members of the PHO regulon provides further evidence that a P redox cycle may have an important role in biology.

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