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# Evidence for Two Phosphonate Degradative Pathways in *Enterobacter aerogenes*

KI-SUNG LEE,† WILLIAM W. METCALF, AND BARRY L. WANNER*

*Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907*

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**We screened mini-Mu plasmid libraries from *Enterobacter aerogenes* IFO 12010 for plasmids that complement *Escherichia coli phn* mutants that cannot use phosphonates (Pn) as the sole source of phosphorus (P). We isolated two kinds of plasmids that, unexpectedly, encode genes for different metabolic pathways. One kind complements *E. coli* mutants with both Pn transport and Pn catalysis genes deleted; these plasmids allow degradation of the 2-carbon-substituted Pn α-aminoethylphosphonate but not of unsubstituted alkyl Pn. This substrate specificity is characteristic of a phosphonatase pathway, which is absent in *E. coli*. The other kind complements *E. coli* mutants with Pn catalysis genes deleted but not those with both transport and catalysis genes deleted; these plasmids allow degradation of both substituted and unsubstituted Pn. Such a broad substrate specificity is characteristic of a carbon-phosphorus (C-P) lyase pathway, which is common in gram-negative bacteria, including *E. coli*. Further proof that the two kinds of plasmids encode genes for different pathways was demonstrated by the lack of DNA homology between the plasmids. In particular, the phosphonatase clone from *E. aerogenes* failed to hybridize to the *E. coli phnCDEFGHIJKLMNOP* gene cluster for Pn uptake and degradation, while the *E. aerogenes* C-P lyase clone hybridized strongly to the *E. coli phnGHIJKLM* genes encoding C-P lyase but not to the *E. coli phnCDE* genes encoding Pn transport. Specific hybridization by the *E. aerogenes* C-P lyase plasmid to the *E. coli phnF*, *phnN*, *phnO*, and *phnP* genes was not determined. Furthermore, we showed that one or more genes encoding the apparent *E. aerogenes* phosphonatase pathway, like the *E. coli phnC*-to-*phnP* gene cluster, is under phosphate regulon control in *E. coli*. This highlights the importance of Pn in bacterial P assimilation in nature.**

Many bacteria can use phosphonates (Pn) as the sole source of phosphorus (P). Since Pn have direct carbon-to-phosphorus (C-P) bonds in place of the carbon-oxygen-phosphorus bond of $P_i$ esters, such bacteria can break C-P bonds. Yet, in spite of the abundance of Pn in nature, very little is understood about the cleavage or biosynthesis of C-P bonds. Natural Pn, such as α-aminoethylphosphonate (AEPn), are widely distributed, especially in the membranes of eucaryotic cells. AEPn is a structural analog of ethanolaminephosphate; in *Tetrahymena* species, it accounts for 30% of the P content of lipids (6). Natural Pn such as the antibiotic fosfomycin are made by *Streptomyces* species. Fosfomycin enters sensitive cells through the glycerol-3-phosphate transporter and once inside inhibits cell wall biosynthesis by acting as an analog of phosphoenolpyruvate in the formation of *N*-acetylmuramic acid residues (2). Like fosfomycin, many synthetic Pn are also analogs of $P_i$ compounds and act as enzyme inhibitors or chemotherapeutic agents. Pn are especially useful inhibitors, in part, because the inherent stability of the C-P bond makes them unusually stable compounds.

There seem to be two metabolic pathways for Pn biodegradation. *Bacillus cereus* and *Pseudomonas aeruginosa* degrade AEPn via a phosphonatase pathway. In a two-step process, AEPn is converted to phosphonoacetaldehyde by an AEPn transaminase and then converted to acetaldehyde and $P_i$ by a phosphonoacetaldehyde hydrolase (trivial name, phosphonatase) (3). C-P bond cleavage by the *B. cereus* phosphonatase involves formation of an aldolaselike imine of phosphonoacetaldehyde with an active-site lysine, which destabilizes the C-P bond (12). Such an enzyme mechanism requires conversion of the α-amino group to an aldehyde before hydrolysis and explains why a phosphonatase cannot act directly on AEPn. Since Pn lacking a 2-carbon substitution cannot be converted to an aldehyde by a transaminase, they cannot be degraded by a phosphonatase pathway. Unfortunately, no genetic information exists on Pn degradation by a phosphonatase pathway.

*Escherichia coli* degrades Pn solely by a C-P lyase that has broad substrate specificity. Both the 2-carbon-substituted Pn AEPn and unsubstituted Pn (such as the alkyl Pn methylphosphonate [MPn] or ethylphosphonate [EPn]) are metabolized by the C-P lyase pathway, which apparently involves direct dephosphonation of the substrate. Since it has been proven difficult to detect the C-P lyase activity in vitro (16), the biochemical mechanism of C-P bond cleavage by a lyase is poorly understood. However, there is now a great deal of genetic and molecular information on Pn degradation by the *E. coli* C-P lyase. All genes encoding Pn uptake and degradation lie in the complex *phnCDEFGHIJKLMNOP* gene cluster, near 93 min (8, 19). The *phnC*, *phnD*, and *phnE* genes probably encode a binding protein-dependent Pn transporter, while most other *phn* gene products are probably needed for catalysis; two genes (*phnF* and *phnO*) may have a regulatory role (9). Also, as expected for genes whose function is to provide cells with an alternative P source, the *E. coli phnC*-to-*phnP* gene cluster is under phosphate (PHO) regulon control (18).

Recently, Murata et al. (10) reported detecting an in vitro C-P lyase activity from *Enterobacter aerogenes* IFO 12010. They partially purified an enzyme, which on the basis of $P_i$ release measurements, apparently acted on both substituted and unsubstituted Pn. The *E. aerogenes* enzyme required

* Corresponding author.
† Permanent address: Department of Biology, Pai-Chai University, Daejeon, Korea.

two protein components and had a specific activity of 3.5 μmol of $P_i$ produced per h per mg of protein and a $K_m$ of 3.5 mM for phosphonoacetate (PnAc). This specific activity is enough to produce more than 1.3 μmol of $P_i$ per generation per mg of protein needed to support a 60-min generation time. However, it should also be mentioned that clear demonstration of an in vitro C-P lyase activity requires the identification of the carbon product. Measurement of $P_i$ release alone is inadequate in the absence of evidence that the $P_i$ was derived from a Pn.

With a goal toward better understanding of Pn metabolism, we cloned *E. aerogenes* IFO 12010 genes that complement *E. coli phn* mutants. Unexpectedly, we found two kinds of complementing plasmids. One kind appears to encode an enzyme(s) in a phosphonatase pathway; the other appears to encode an enzyme(s) in a C-P lyase pathway. This is the first demonstration of two separate Pn degradative pathways in the same cell. Biochemical data for two pathways are lacking because of the absence of a reliable method to detect C-P lyase activity in vitro. Therefore, past studies have generally assumed single metabolic pathways (16). We also show that the genes encoding phosphonatase and C-P lyase Pn degradative pathways in *E. aerogenes* are unrelated.

## MATERIALS AND METHODS

**Media and chemicals.** Most media and chemicals were the same as described previously (8, 17, 19). 5-Bromo-4-chloro-3-indolylphosphate-*p*-toluidine (X-P) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside from Bachem (Torrance, Calif.) are the blue dyes for detecting β-galactosidase and bacterial alkaline phosphatase (Bap) activities, respectively; both were used as described previously (17). [α-$^{32}$P]dATP was from Amersham Corp. (Arlington Heights, Ill.). Dimethylphosphite (DMPt) was from Aldrich (Milwaukee, Wis.). Trimethoprim was from Sigma (St. Louis, Mo.).

Tryptone-yeast extract (TYE) and glucose M63 or glucose-morpholinepropane sulfonic acid (MOPS) media were used routinely as complex and minimal media, respectively. Solid media contained 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Ampicillin and streptomycin were added at 100 μg/ml, chloramphenicol was added at 25 μg/ml, and kanamycin was added at 50 μg/ml; tetracycline was added at 15 μg/ml to TYE agar with 2.5 mM $PP_i$. Trimethoprim was added at 100 or 400 μg/ml.

**Bacteria, phages, and plasmids.** *E. aerogenes* IFO 12010 (ATCC 15038) was from American Type Culture Collection (Rockville, Md.). CT152 [Mu *c62* ($Ts^-$) *pf7701* Δ*455-3 kan*) was from M. Howe (15); pEG5166 in MC1040 [Mu *c62* ($Ts^-$)], pREG2-1 in M8820 [Δ(*ara leu*) DE5 (*proBA lac*) *rpsL*], and pRK24M4 in QSR17 (*thr leu trpE*) were from E. Groisman; pRK24 in DF4063 (*thr leu*) was from M. Casadaban (5). All *E. coli* strains are derivatives of *E. coli* K-12; the *phn*(EcoB) locus is from *E. coli* B (19). Unlike the *E. coli* K-12 *phn*(EcoK) locus, which is cryptic, the *phn*(EcoB) locus confers a $Pn^+$ phenotype (19). *E. coli* strains made in this laboratory are described in Table 1.

The mini-Mu cloning vector pEG5166 is an $Amp^r$ ColE1 replicon with an insertion of the mobilizable ($Mob^+$) $Cm^r$ replicon Mu dI5166; pREG2-1 is a $Tmp^r$, transfer-proficient ($Tra^+$) descendant of the IncW plasmid R388 with an insertion of Mu *c62* ($Ts^-$) Ap1; pRK24 is an $Amp^r$ $Kan^s$ $Tc^r$ $TrpE^+$ $Tra^+$ descendant of the IncP plasmid RP4 (RK2 and RP1); and pRK24M is pRK24 with a Mu *c62* ($Ts^-$) insertion (5). pBW120 and the mobilization helper plasmid pRK2013 were described previously (19, 20). pBW120 is $Amp^r$, and pRK2013 is $Kan^r$; both are ColE1 replicons.

**Molecular genetics.** Transductions with P1*kc*, conjugations, and DNA transformations were done as previously described (17, 19).

**Molecular biology procedures.** Plasmid and chromosomal DNAs were isolated as described previously (19). Restriction enzymes were used according to suppliers' recommendations. DNAs were separated on 0.7% agarose gels and transferred to GeneScreen (Du Pont Co., Wilmington, Del.). The membranes were probed with randomly primed [α-$^{32}$P]dATP-labeled DNAs, as described previously (19).

**Bap assays.** Bap activities were measured as described previously (17). Units are nanomoles of product made per minute at 37°C. Cell culture optical density at 420 nm ($OD_{420}$) was measured.

**Phenotypic tests and growth studies.** P compounds were filter-sterilized and, unless noted otherwise, were added to glucose-MOPS medium at 0.1 mM in place of $P_i$. Plate tests for use of a compound as the sole P source were done by streaking cells side-by-side on glucose-MOPS agar together with suitable control strains. $Pn^+$ control strains included *E. aerogenes* IFO 12010 and *E. coli* BW14332; $Pn^-$ control strains included *E. coli* BW14329, BW14893, and BW16787. All positive growth tests were verified by OD measurements of glucose-MOPS liquid cultures. In this study, both *E. aerogenes* and *E. coli* were grown at 30°C when a minimal medium was used. This is because *E. aerogenes* grew poorly on minimal medium at 37°C and not at all at 42°C; we found that *E. aerogenes* shows a temperature-sensitive ($Ts^-$) methionine auxotrophy, similar to some other strains of the family *Enterobacteriaceae* (13).

**Construction of *E. aerogenes* mini-Mu cloning host.** The application of mini-Mu phage cloning to *E. aerogenes* required the construction of suitable Mu *c62* ($Ts^-$) lysogens which were made by mating *E. aerogenes* with M8820 (pREG2-1, $Leu^-$ $Pro^-$). Of 20 $Tmp^r$ *E. aerogenes* exconjugants, all were $Ts^-$ and released Mu, as determined by stabbing colonies into a lawn of the $HsdR^-$ *E. coli* BW4714. In contrast, no $Kan^r$ $Ts^-$ *E. aerogenes* transductants made with the Mu *c62* ($Ts^-$) *pf7701 kan* phage (from CT152) or $Tc^r$ *E. aerogenes* exconjugants made with pRK24M released phage. We do not know why pREG2-1 exconjugants released Mu, while similar ones made with other phages did not.

pEG5166S was crossed into BW16658 [*E. aerogenes* (pREG2-1)] in a tripartite mating with BW7308 (pRK2013 $Ilv^-$ $His^-$ $Trp^-$) and BW16657 (pEG5166S $Ilv^-$ $His^-$ $Trp^-$ $Pro^-$). Four independent $Tmp^r$ $Cm^r$ *E. aerogenes* exconjugants, BW16662 to BW16665, were isolated as $Cm^r$ $Tmp^r$ prototrophic exconjugants of *E. aerogenes*. [pEG5166S is itself an apparent Mu dI5166 mini-Mu clone that is $Amp^s$; its structure, on the basis of restriction mapping, was indistinguishable from that of pEG5166, however (data not shown). BW16657 harboring pEG5166S was isolated as a $Cm^r$ $Amp^s$ transformant of BW11442 with plasmid DNA of MC1040 (pEG5166).]

**Molecular cloning of *E. aerogenes* DNA.** Mini-Mu plasmid libraries in the form of phage lysates were made by a modification of a procedure for the preparation of Mu lysates from *E. aerogenes* (11). Our protocol involved heat induction of *E. aerogenes* exconjugants BW16662 to BW16665, which harbored both pREG2-1 and pEG5166S as follows. The donor cells were grown with shaking in baffled flasks at 30°C in chloramphenicol- and trimethoprim-containing LB liquid medium to an $OD_{420}$ of 0.6. The cultures were made

TABLE 1. Bacterial strains

| Strain[a] | Genotype | Pedigree | Source or reference |
|---|---|---|---|
| BW2930 | Δ*lac-169* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 aroB crp-72 rpsL267 thi* | XPh1a via BW1470 (19) | Spontaneous Ts$^{+b}$ |
| BW3739 | Δ*lac-169 thr*::Tn*5 creB510* | BD792 via BW3414 (19) | Kan$^r$ with P1 on DB6980 (17) |
| BW4714 | *lac-169 phn*(EcoK)$^c$ *creB510 hsdR514* | BD792 via BW3739 | 19 |
| BW5104 | Mu-1 Δ*lac-169 creB510 hsdR514* | BD792 via BW4714 | Lysogenized with Mu-1 |
| BW7308 | pRK2013/IN (*rrnD-rrnE*)*1 ilv-1 his-29*(Am) *trpA9605*(Am) *trpR tsx ara malT* | FE103 via BW358 920) | Kan$^r$ exconjugant with RR1/pRK2013 |
| BW9766 | Δ*lac-169* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 THI* | XPhla via BW2930 (19) | Aro$^+$ with P1 on BW3912 (19) |
| BW9767 | Δ*lac-169* Δ*lac-169* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 rpsL267 thi* | XPhla via BW2930 | Aro$^+$ with P1 on BW3912 |
| BW11083 | Mu-1 *zjd-1*::Tn*10* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 hsdR514* | BD792 via BW5104 (7) | Tc$^r$ with P1 on BW10197 (19) |
| BW11441 | Mu-1 Δ*lac-169* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 creB510 thi* | XPhla via BW9766 (19) | Lysogenized with Mu-1 |
| BW11442 | Mu-1 *lac-169* IN(*rrnD-rrnE*)*1 ilv-1 his-29*(Am) *trpA9605*(Am) *trpR tsx ara malT proC* | FE103 via BW453 (19) | Lysogenized with Mu-1 |
| BW12918 | DE3 (*lac*)X74 Δ*phoA532 phnP2*::Tn*5-132*(*tet*) | MG1655$_{MC}$ via BW12720 (8) | Tc$^r$ with P1 on BW12466 (8) |
| BW13197 | DE3(*lac*)X74 *phnP2*::Tn*5-112* Δ*phoA532* | Mg1655$_{MC}$ via BW12918 | Kan$^r$ Tc$^s$ with λ::Tn*5-112* |
| BW13249 | DE3(*lac*)X74 Δ(*phoBR*)*525* | MG1655$_{MC}$ via BW13183 (8) | Pro$^+$ with P1 on BW13034 (8) |
| BW13746 | DE3 (*lac*)X74 Δ(*phoBR*)*525* | BD792 via BW13635 (8) | Pro$^+$ with P1 on BW13249 |
| BW14277 | Mu-1 Δ*lac-169 proC*::Tn*5-132*(*tet*) *creB510 hsdR514* | BD792 via BW5104 | Tc$^r$ with P1 on BW10244 (7) |
| BW14295 | Mu-1 Δ*lac-169* Δ*phoA532 creB510 hsdR514 phn*(EcoK) | BD792 via BW14277 | Pro$^+$ with P1 on BW13247 (8) |
| BW14329 | Mu-1 Δ*lac-169* Δ(*mel proP phnCDEFGHIJKLMNOP*)*2*::Tn*5* seq*1/132*(*tet*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14295 | Tc$^r$ with P1 on BW14001 (8) |
| BW14332 | DE3 (*lac*)X74 *phn*(EcoB)$^c$ Δ*phoA532* | BD792 via BW14331 (8) | Mel$^+$ with P1 on BW10748 (19) |
| BW14649 | Mu-1 Δ*lac-169* Δ(*phoBR*)*525 creB510 hsdR514* | BD792 via BW14277 | Pro$^+$ with P1 on BW13746 |
| BW14893 | DE3 (*lac*)X74 Δ*phoA532* Δ(*phnC*?DEFGHIJKLMNOP)*33-30* | BD792 via BW14331 | Mel$^+$ with P1 on BW9766 (19) |
| BW15191 | DE3 (*lac*)X74 Δ(*mel proP phnCDEFGHIJKLMNOP*)*2*::Tn*5*seq *1/132*(*tet*) | BD792 via BW13711 (7) | Tc$^r$ with P1 on BW14001 |
| BW15268 | DE3 (*lac*)X74 *phn*(EcoB) | BD792 via BW15191 | Mel$^+$ with P1 on BW10748 |
| BW16627 | *E. aerogenes rpsL* | IFO 12010 | Spontaneous Str$^r$ |
| BW16657 | pEG5166S/Mu-1 Δ*lac-169* IN(*rrnD-rrnE*)*1 ilv-1 his-29*(Am) *trpA9605*(Am) *trpR tsx ara malT proC* | FE103 via BW11442 | Cm$^r$ transformants |
| BW16658 | *E. aerogenes*/pREG2-1 | IFO 12010 | Tmp$^r$ exconjugant with pREG2-1/M8820 |
| BW16662 to BW16665 | *E. aerogenes*/pEG5166S, pREG2-1 | IFO 12010 via BW16658 | Cm$^r$ exconjugant with BW16657 and BW7308 |
| BW16685 | Mu-1 Δ*lac-169 phnE1*::Tn*5-112*(*kan*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14329 | Mel$^+$ with P1 on BW13196 (9) |
| BW16686 | Mu-1 Δ*lac-169 phnP3*::Tn*5-112*(*kan*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14329 | Mel$^+$ with P1 on BW13199 (8) |
| BW16687 | Mu-1 Δ*lac-169 phnH4*::Tn*5-112*(*kan*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14329 | Mel$^+$ with P1 on BW13201 (8) |
| BW16688 | Mu-1 Δ*lac-169 phnK6*::Tn*5-112*(*kan*) Δ*phoA532 creB510 hsdR514* | BD792 via BW14329 | Mel$^+$ with P1 on BW13204 (8) |
| BW16689 | Mu-1 Δ*lac-169 phnJ7*::Tn*5-112*(*kan*) Δ*phoA532 creB510 hsdR514* | BE792 via BW14329 | Mel$^+$ with P1 on BW13205 (8) |
| BW16706 | DE3(*lac*)X74 *phnH4*::Tn*5-132*(*tet*) *phnP2*::Tn*5-112*(*kan*) Δ*phoA532* | MG1655$_{MC}$ via BW13197 | Tc$^r$ with P1 on BW12479 (8) |
| BW16711 | DE3 (*lac*)X74 Δ*phoA532* Δ(*phnHIJKLMNOP*)*4*/2::Tn*5-112*(*kan*) | MG1655$_{MC}$ via BW16706 | Spontaneous Tc$^s$ |
| BW16716 | DE3(*lac*)X74 *phoA532* Δ(*phnHIJKLMNOP*)*4*/2::Tn*5-132*(*tet*) | MG1655$_{MC}$ via BW16711 | Tc$^r$ Kan$^s$ swap with λ::Tn*5-132* (*) |
| BW16787 | Mu-1 Δ*lac-169* Δ(*phnHIJKLMNOP*)*4*/2::Tn*5-132*(*tet*) Δ(*phoA532 creB510 hsdR514* | BD792 via BW14329 | Mel$^+$ with P1 on BW16716 |

[a] All strains are *E. coli* K-12 unless noted otherwise.
[b] Ts$^+$, temperature insensitive.
[c] The *phn* loci differ in *E. coli* K-12 and *E. coli* B and are denoted *phn*(EcoK) and *phn*(EcoB), respectively. The *phn*(EcoK) allele is mutable to Pn$^+$, while the *phn*(EcoB) allele is naturally Pn$^+$ (19). All *phn*::Tn*5* mutations were made in strains with the *phn*(EcoB) allele (8).

0.2 M $MgSO_4$, shifted to a shaking water bath at 43°C for 30 min, and then to a shaking water bath at 37°C for about 1 h. The cells were treated with chloroform. Debris was removed by centrifugation. The resulting lysates were used within 1 to 2 h of preparation which was necessary because of the extreme instability of *E. aerogenes* mini-Mu lysates, in comparison with similar ones of *E. coli*. Newly made lysates gave thousands of Cm$^r$ transductants per 10 μl immediately after preparation; the same lysates gave no Cm$^r$ transductants when tested similarly 12 to 24 h later.

Cm$^r$ mini-Mu were selected after transduction of the Mu-1 lysogenic HsdR$^-$ *E. coli* mutant BW11083 [Δ(*phnCDEFG*

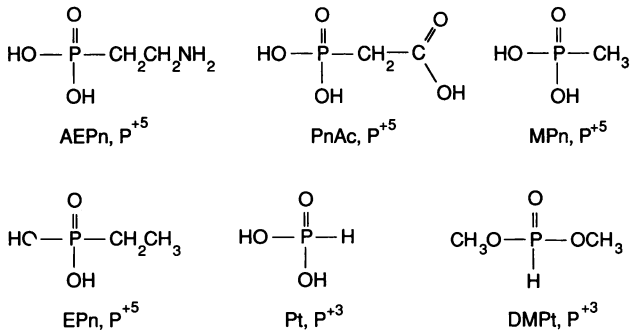


FIG. 1. Structures of P compounds. AEPn, PnAC, MPn, EPn, Pt, and DMPt are described in the text. The P oxidation state in each compound is shown.

*HIJKLMNOP*)], BW14329 [Δ(*phnCDEFGHIJKLMNOP*) Δ*phoA*], or BW16787 [Δ(*phnHIJKLMNOP*) Δ*phoA*]. Several thousand transductants were replica plated onto glucose-MOPS agar with AEPn, DMPt, EPn, MPn, phosphite (Pt), or *O*-phosphoserine (Fig. 1) as the sole P source. About 0.5% of the transductants grew on a P source on which the parental strain failed to grow. $Cm^r$ transductants were also selected on TYE-chloramphenicol agar with X-P, on which $X\text{-}P^+$ transductants were identified as blue colonies. $Pn^+$ plasmids were isolated and transformed into BW11441 for further testing.

## RESULTS

**Pn utilization in *E. coli* and *E. aerogenes*.** *E. aerogenes* IFO 12010 appeared to grow equally well on glucose-MOPS agar with AEPn, DMPt, EPn, MPn, PnAc, and Pt as with $P_i$ as the sole P source. In contrast, *E. coli* BW14332 grew best with AEPn, MPn, and $P_i$; it grew noticeably slower with EPn and much slower with Pt. *E. coli* BW14332 grew very poorly on PnAc and barely or not at all on DMPt. (See Fig. 1 for the chemical structures of various Pn and related P compounds tested.) We also compared growth on P sources in liquid cultures. To do this, we inoculated *E. coli* and *E. aerogenes* into glucose-MOPS medium with limiting or excess $P_i$ or with limiting $P_i$ plus excess AEPn, MPn, or Pt. We used $phoA^+$ *E. coli* BW15268 so that we could follow Bap synthesis as an indicator of induction of the PHO regulon; *E. aerogenes* carries the $phoA^+$ gene. We sampled cultures for growth and Bap activities continuously over a 28-h period (Fig. 2).

Our data on liquid cultures are in full agreement with the growth observed on glucose-MOPS agar. *E. coli* grew with a μ value of 0.5 doublings per h on $P_i$ at 30°C; it grew with a μ value of 0.4 on AEPn or MPn and with a μ value of 0.3 on Pt. Interestingly, *E. coli* showed a distinct diauxic growth pattern upon $P_i$ limitation in medium with added AEPn, MPn, or Pt. *E. coli* made Bap at a rate of 1.0 U per $OD_{420}$ when $P_i$ was in excess; it made Bap at a rate of about 290 U per $OD_{420}$ in all $P_i$-limited cultures above an $OD_{420}$ of 0.1, which is when the $P_i$ (0.0125 mM) in the medium is expected to be exhausted.

*E. aerogenes* always grew faster than *E. coli*. *E. aerogenes* grew with a μ value of 0.9 doublings per h on $P_i$; it grew with a μ value of 0.9 on AEPn and with a μ value of 0.5 on MPn or Pt. Like *E. coli*, *E. aerogenes* showed a distinct diauxic growth pattern upon $P_i$ limitation in medium with MPn or Pt. But, unlike *E. coli*, *E. aerogenes* showed no diauxie effect in AEPn medium. In *E. aerogenes*, Bap synthesis was induced near an $OD_{420}$ of 0.1 in all $P_i$-limited cultures. *E. aerogenes* made Bap at a rate of 0.4 U per $OD_{420}$ when $P_i$ was in excess; it initially made Bap at a rate over 100 U per $OD_{420}$ in all $P_i$-limited cultures; it continued to synthesize Bap at a higher rate of about 290 U per $OD_{420}$ in MPn and Pt media. However, following an initial period of rapid synthesis, *E. aerogenes* synthesized Bap at the lower rate of only 16.5 U per $OD_{420}$ during steady-state growth on AEPn (Fig. 2D). Both the absence of a diauxie effect during the shift from $P_i$ to AEPn utilization and the ability of *E. aerogenes* to (partially) repress Bap synthesis during growth on AEPn were unexpected. These data suggest that *E. aerogenes* may use different pathways for metabolism of AEPn than for metabolism of MPn or Pt.

**Cloning of *E. aerogenes* phosphonatase and C-P lyase genes by complementation of *E. coli phn* mutants.** We previously cloned large DNA fragments of the *E. coli phnC*-to-*phnP* gene cluster by complementation of a Δ(*phnC?DEFGHI JKLMNOP*)*33-30* mutant, which we later proved had nearly the entire *phnC*-to-*phnP* gene cluster deleted (Fig. 3). Such $Pn^+$ *E. coli* mini-Mu plasmid clones were selected on glucose-MOPS-MPn agar (19). They also complemented Δ(*phnC?DEFGHIJKLMNOP*)*33-30* and Δ(*phnCDEFGHI JKLMNOP*)*2* mutants for use of AEPn, EPn, MPn, or Pt as the sole P source. In addition, the subclone pBW120 complemented a Δ*phoA* Δ(*phnCDEFGHIJKLMNOP*) mutant for use of X-P or *O*-phosphoserine as the sole P source. This phosphate ester-positive phenotype is probably due to nonspecific transport of X-P or *O*-phosphoserine by the *phnCDE* Pn transporter, because growth on X-P or *O*-phosphoserine requires also a cytoplasmic phosphatase(s). A blue color on X-P agar and growth on *O*-phosphoserine agar are indicators of an intact *E. coli* Pn transporter or the *phoA* gene (8). No effect on use of X-P or *O*-phosphoserine is seen in $phoA^+$ Δ(*phnCDEFGHIJKLMNOP*) hosts, since Bap ordinarily hydrolyzes such phosphate esters in the periplasm.

We used the mini-Mu phage technique to clone *E. aerogenes* genes because such plasmid libraries have the capacity to carry large DNA segments. We expected that the *E. aerogenes* genes for Pn utilization were similar in size to the *E. coli phnC*-to-*phnP* gene cluster. Unexpectedly, all attempts to select $Pn^+$ plasmids from *E. aerogenes* libraries failed when growth of $HsdR^-$ *E. coli* Δ(*phnC?DEFGHI JKLMNOP*)*33-30* and Δ(*phnCDEFGHIJKLMNOP*)*2* mutants was selected on glucose-MOPS agar with DMPt, EPn, MPn, or Pt as the sole P source. $Pn^+$ plasmids from *E. aerogenes* libraries were obtained by complementation of these mutants only when growth was selected on glucose-MOPS-AEPn agar. Also, of 48 $AEPn^+$ plasmids that complemented these hosts for growth on glucose-MOPS-AEPn agar, none supported growth of *E. coli* on DMPt, EPn, MPn, or Pt. Sixteen plasmid DNAs examined by restriction mapping were different, yet each had one or more (nonvector) bands in common when digested with different enzymes. Thus, each probably contained the same chromosomal locus.

We also isolated $AEPn^+$ transductants of the Δ*phoA* Δ(*phnCDEFGHIJKLMNOP*)*2* host. Of eight $AEPn^+$ transductants, none was blue on X-P agar or grew on glucose MOPS *O*-phosphoserine agar. Thus, the $AEPn^+$ plasmids do not appear to encode a Pn transporter like the one in *E. coli*. Conversely, none of 12 transductants selected as a blue colony on X-P agar was $AEPn^+$, $Bap^+$, or grew with any other Pn as the sole P source. It is not surprising that none of

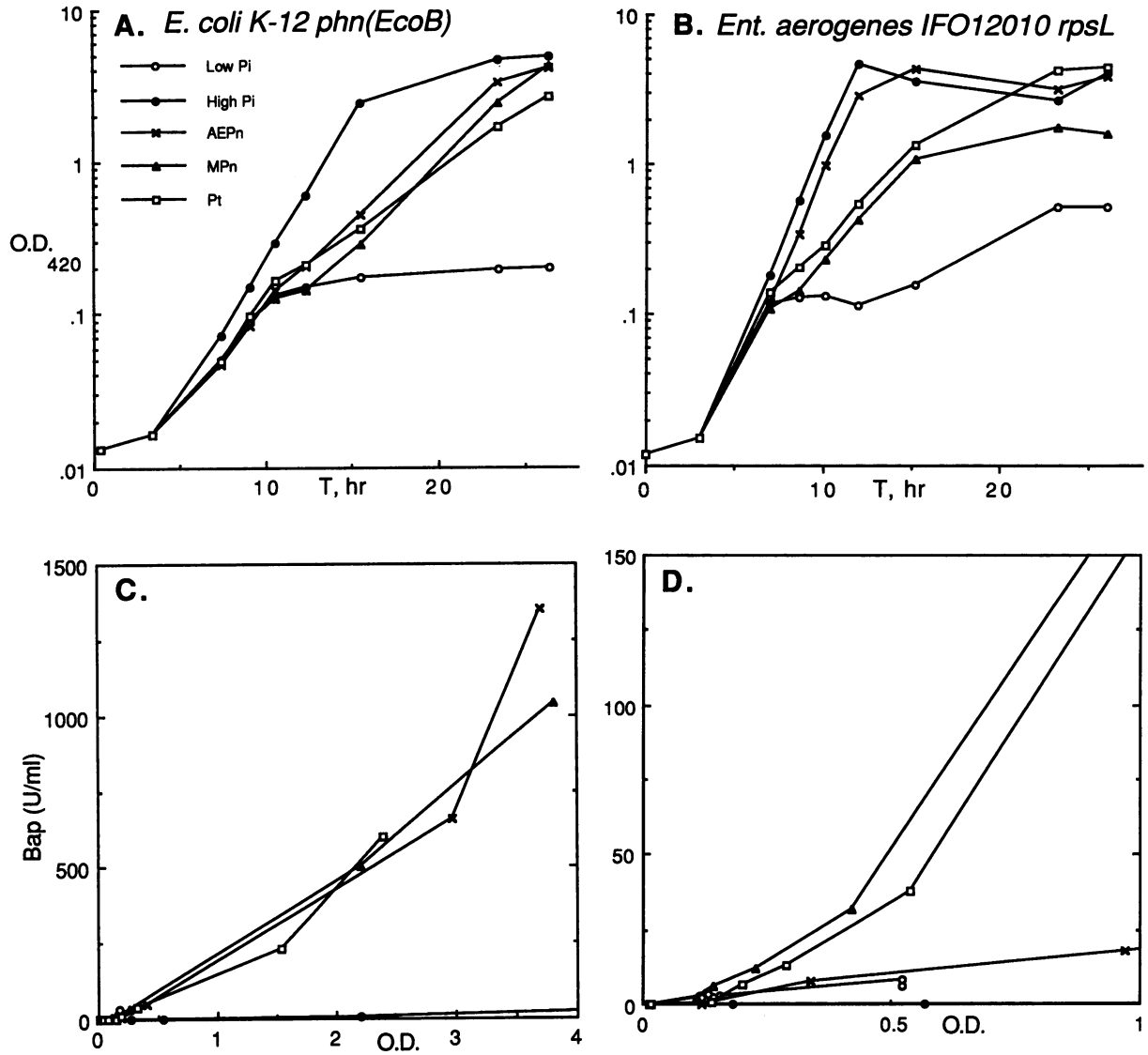


FIG. 2. Growth of *E. coli* and *E. aerogenes* on different P sources. Cultures were grown at 30°C and sampled periodically for measurements of turbidity and Bap activity, as described previously (21). (A) *E. coli* K-12 *phn*(EcoB) BW15268 in glucose-MOPS medium with limiting (0.0125 mM) or excess (0.5 mM) $P_i$ or with limiting $P_i$ plus 0.5 mM of an alternative P source. (B) *E. aerogenes* BW16627 in the same medium. (C) Differential plot of Bap synthesis in *E. coli* cultures. (D) Differential plot of Bap synthesis in *E. aerogenes* cultures. The scale in panel D was expanded to show enzyme derepression in the AEPn culture; lines are continuous with data points off the scale. Colonies were pregrown on glucose–MOPS–0.1 mM $P_i$ agar for 24 h. Cells were suspended in 0.85% saline and inoculated into 25 ml of 0.4% glucose–MOPS medium with 0.0125 mM $P_i$ or 0.0125 mM $P_i$ plus 0.5 mM $P_i$, AEPn, MPn, or Pt. Cultures were aerated by shaking in 250-ml baffled flasks. Data from single experiments are plotted. Numerical values cited in the text are the averages of two separate growth experiments in most media.

12 X-P positive clones was $Bap^+$, because X-P is a rather nonspecific phosphatase substrate.

We considered the idea that, unlike the *E. coli phnC*-to-*phnP* gene cluster, the *E. aerogenes* genes coding for use of DMPt, EPn, MPn, or Pt were dispersed. In particular, we considered that genes coding for Pn transport and C-P bond cleavage were unlinked. Therefore we selected $Pn^+$ plasmids by complementation of the *E. coli* $HsdR^-$ Δ(*phnHI JKLMNOP*)*4/2* mutant, which has a functional Pn transporter but has most of the catalysis genes coding for C-P lyase deleted. In *E. coli*, loss of either a Pn transport or a C-P lyase catalysis gene simultaneously abolishes the use of Pn as well as Pt as the sole P source (8).

$Pn^+$ plasmids from *E. aerogenes* libraries were obtained by complementation of the Δ(*phnHIJKLMNOP*) host on glucose-MOPS agar with AEPn, DMPt, EPn, MPn, and Pt as the sole source of P. Also, with this mutant 2 of 20 transductants selected on AEPn agar grew on all five P sources; 18 grew only on AEPn. Thirty transductants were purified from each selective medium and tested on the other P sources. Of the $DMPt^+$, $EPn^+$, $MPn^+$, and $Pt^+$ transductants, 85% grew on all five P sources, including AEPn; the other 15% were unstable, because after purification many failed to grow even on the P source on which it had originally been selected. Therefore, we obtained two kinds of plasmids that allow for use of AEPn as the sole P source: one kind

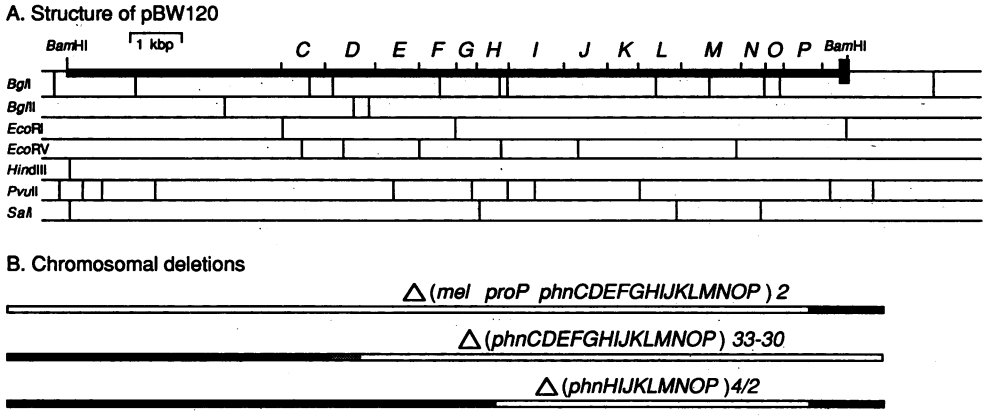


FIG. 3. Structure of the *phnC*-to-*phnP* gene cluster in *E. coli*. (A) Structure of pBW120. Fourteen genes named, in alphabetical order, *phnC*-to-*phnP* are transcribed rightward, which is counterclockwise on the *E. coli* map. The top line shows the 15.6-kb *Bam*HI fragment that was subcloned to make pBW120 (19) and that was sequenced (1). The left *Bam*HI site is a chromosomal site; the right *Bam*HI site is in vector DNA (19). The lower lines show the corresponding chromosomal restriction fragments for seven enzymes, based on the DNA sequence, the *E. coli* restriction map, and DNA hybridization (1, 19). (B) The sizes of the chromosomal deletions complemented are illustrated. Solid bars show *E. coli* DNA; open bars show the extents of the Δ(*phnCDEFGHIJKLMNOP*)*2*, Δ(*phnC*?*DEFGHIJKLMNOP*)*33-30*, and Δ(*phnHIJK LMNOP*)*4/2* deletions, which are also called the Δ(*mel*-to-*phnP*)*2*::Tn*5*seq*1/132*, Δ(*phnC*?*DEFGHIJKLMNOP*)*33-30*, and Δ(*phnHIJKLMN OP*)*4/2*::Tn*5-132* deletions, respectively. The stippled bar shows the approximate endpoint of the Δ(*phnC*?*DEFGHIJKLMNOP*)*33-30* deletion. The Δ(*phnCDEFGHIJKLMNOP*)*2* deletion arose by homologous recombination of Tn*5* elements in a *mel*::Tn*5*seq*1 phnP2*::Tn*5-132* mutant (8). The Δ(*phnC*?*DEFGHIJKLMNOP*)*33-30* deletion arose by excision of the Mu dl element in the *phnD* (*psiD*)*33*::*lacZ* (Mu dl) mutant; it removes about 20 kb of DNA from near the 3′ end of the *phnC* gene to beyond the *phnP* gene; its left endpoint lies within the shaded region (19). The Δ(*phnHIJKLMNOP*)*4/2* deletion arose by recombination of Tn*5* elements in a *phnH4*::Tn*5 phnP2*::Tn*5-132* mutant (8).

allows use only of AEPn, whereas the other kind allows use of a broad spectrum of Pn, including AEPn, EPn, and MPn. The latter plasmids also allow use of DMPt or Pt. Five complemented hosts grew with a μ of 0.3 to 0.4 on MPn or Pt and with a μ of 0.2 on DMPt (data not shown). Restriction mapping showed that all $Pn^+$ plasmids were at least 27 kbp long. Because the vector Mu dI5166 is 15.8 kbp long (4), all had chromosomal inserts of greater than 11 kbp (data not shown).

**Complementation of *E. coli phn* mutants.** We considered that the two kinds of $AEPn^+$ plasmids differed simply by the presence or absence of a gene(s) for an AEPn-specific transporter. However, subsequent studies ruled out this possibility and instead suggested that the two kinds of plasmids encode genes for two different Pn degradative pathways. Plasmids that complemented for growth only on AEPn probably encode genes for a phosphonatase pathway, which is absent in *E. coli*. Such plasmids probably also encode an AEPn-specific transporter, because they complemented Δ(*phnCDEFGHIJKLMNOP*) mutants as well. Plasmids that complemented for growth on AEPn, DMPt, EPn, MPn, and Pt probably encode genes for a C-P lyase pathway, like the one in *E. coli*. Such plasmids do not encode a transporter because they complemented only Δ(*phnHIJK LMNOP*) mutants and not Δ(*phnCDEFGHIJKLMNOP*) mutants, which lack Pn transport genes. Results that support these conclusions are summarized below.

We tested representative plasmids for complementation of a series of *E. coli phn* mutants in order to determine whether or not the two kinds were related. Table 2 shows that *E. aerogenes* uses AEPn, DMPt, EPn, MPn, Pt, or *O*-phosphoserine as the sole P source; except for DMPt, *E. coli* uses the same P sources. Importantly, the use of AEPn, EPn, MPn, or Pt by *E. coli* requires both transport and catalysis genes in the *phnC*-to-*phnP* gene cluster; the use of *O*-phosphoserine or X-P by an *E. coli phoA* mutant requires only the *phnC*, *phnD*, and *phnE* Pn transport genes, in addition to an un-

TABLE 2. Complementation of *E. coli phn* mutants

| Host[a] | Plasmid | Growth on glucose-MOPS agar with the following P source[b]: | | | | | |
|---|---|---|---|---|---|---|---|
| | | AEPn | DMPt | EPn | MPn | Pt | P-Ser |
| *E. aerogenes* | None | + | + | + | + | + | + |
| *E. coli* | | | | | | | |
| Wild type | None | + | − | + | + | + | + |
| *phnE*[c] | None | − | − | − | − | − | − |
| *phnE* | pKL38[d] | + | − | − | − | − | − |
| *phnE* | pKL124[e] | − | − | − | − | − | − |
| Δ(*phnC-P*) | None | − | − | − | − | − | − |
| Δ(*phnC-P*) | pKL38 | + | − | − | − | − | − |
| Δ(*phnC-P*) | pKL124 | − | − | − | − | − | − |
| Δ(*phnH-P*)[f] | None | − | − | − | − | − | + |
| Δ(*phnH-P*) | pKL38 | + | − | − | − | − | + |
| Δ(*phnH-P*) | pKL124 | + | + | + | + | + | + |
| Δ(*phoBR*) | None | − | − | − | − | − | − |
| Δ(*phoBR*) | pKL38 | − | − | − | − | − | − |

[a] *E. coli* strains included BW14322 [$Pn^+$ *phn*(EcoB) Δ*phoA*], BW16685 (*phnE* Δ*phoA*), BW14329 [Δ(*phnC*?*DEFGHIJKLMNOP*) Δ*phoA*], BW16787 [Δ(*phnHIJKLMNOP*) Δ*phoA*], and BW14649 [Δ(*phoBR*)].

[b] + growth; −, no growth. P-Ser, *O*-phosphoserine.

[c] The *phnE* mutation in BW16685 is due to a Tn*5-112* insertion, which even though it may cause polarity, gives rise to nonpolar *phnE*::Tn*5-112* insertions at a high frequency as a result of recombinational switching (8). Therefore, an inability to complement this *phnE* mutant shows a requirement for *phnE* and not for some downstream gene.

[d] The phosphonatase clones pEG5166-KL38 and pEG5166-KL39 gave identical results.

[e] The C-P lyase clones pEG5166-KL107, pEG5166-KL108, pEG5166-KL115, pEG5166-KL119, pEG5166-KL124, and pEG5166-KL127 gave identical results.

[f] The *phnH* mutant BW16687, the *phnJ* mutant BW16689, the *phnK* mutant BW16688, and the *phnP* mutant BW16686 gave results identical to those of the Δ(*phnHIJKLMNOP*) mutant BW16787, in complementation tests with all plasmids. Like the *phnE*::Tn*5-112* mutant described above, the *phnH*, *phnJ*, *phnK*, and *phnP* mutations are due to a Tn*5-112* insertion and should have therefore given rise to $Pn^+$ recombinants by inversion of the element, if such recombinants were able to grow (8).

linked gene for a cytoplasmic phosphatase(s) (8). The phosphonatase plasmids, e.g., pEG5166-KL38, complemented all *phn* mutants for use of AEPn, but they failed to complement any *phn* mutant for use of DMPt, EPn, MPn, Pt, *O*-phosphoserine, or X-P (Table 2). Since the *E. coli phnCDE* transporter allows uptake of EPn, MPn, Pt, *O*-phosphoserine, or X-P, a plasmid with Pn catalysis genes and lacking transport genes is expected to complement Δ(*phnHIJKLM NOP*) mutants for degradation of Pn substrates. Therefore, the phosphonatase plasmids encodes catalysis genes for the specific degradation of AEPn. Also, while the phosphonatase plasmids probably does encode a Pn transporter, that transporter is apparently also AEPn specific, because the phosphonatase plasmid fails to complement the *phnE* transport mutant for use of alternative P sources.

The C-P lyase-positive plasmids with *E. aerogenes* DNA, e.g., pEG5166-KL124, complemented the Δ(*phnHIJKLM NOP*) mutant for use of AEPn, DMPt, EPn, MPn, and Pt, but they failed to complement the Δ(*phnCDEFGHIJKLM NOP*) or *phnE* mutant (Table 2). Therefore, the inability to complement the latter is probably due to the absence of transport genes on plasmids with genes for the (apparent) *E. aerogenes* C-P lyase. Our failure to isolate plasmids with transport genes together with C-P lyase genes from a large library of *E. aerogenes* DNA implies that transport and C-P lyase genes are unlinked in *E. aerogenes*.

In *E. coli*, the *phnC*-to-*phnP* gene cluster is under PHO regulon control. Since the phosphonatase plasmid, pEG 5166-KL38, failed to complement a Δ(*phoBR*) mutant, at least one gene for the apparent phosphonatase pathway is probably also under PHO regulon control (20) and requires the PHO regulon activator PhoB for expression. We could not determine the basis for the lack of complementation of a *phoBR* mutant by a plasmid for the *E. aerogenes* C-P lyase, because complementation by such plasmids required the *phnCDE* genes, which are themselves under PHO regulon control. However, genes encoding the *E. aerogenes* C-P lyase are probably also under PHO regulon control, because they are homologous to ones under PHO regulon control in *E. coli* (see below).

**Comparison of *E. aerogenes* and *E. coli phn* genes.** The above data show that we isolated two kinds of plasmids with *E. aerogenes* genes for use of Pn as the sole P source. To determine whether the genes were related, we tested for homology between both kinds of plasmids and *E. coli* genes for Pn utilization. We compared the DNAs in several ways. We examined pBW120 which carries the *E. coli phnC*-to-*phnP* gene cluster, pEG5166-KL38 which carries a gene(s) for the *E. aerogenes* phosphonatase pathway, pEG5166-KL124 which carries a gene(s) for the *E. aerogenes* C-P lyase pathway, and the vector pEG5166 by using the same four plasmids as probes. We also examined chromosomal DNAs for hybridization with the $Pn^+$ plasmids.

Both the *E. aerogenes* and *E. coli* C-P lyase plasmids had one or more nonvector DNA bands that hybridized to each other for four restriction enzymes tested. Specific bands that do not contain vector sequences that correspond to the *E. coli phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, and *phnM* genes were detected by the *E. aerogenes* plasmid pEG5166-KL124 (Fig. 4). This shows that the *E. aerogenes* genes we cloned encode a C-P lyase, as predicted above. Because no *Bgl*I, *Pvu*II, *Eco*RV, or *Eco*RI bands specific for the *phnF*, *phnN*, *phnO*, and *phnP* genes in pBW120 were observed (Fig. 3), hybridization to these genes was not established. Therefore, the pEG5166-KL124 plasmid includes genes homologous to the *phnG*-to-*phnM* region; it may also include the *phnF*, *phnN*, *phnO*, and *phnP* genes. In contrast, the phosphonatase plasmid hybridized only to bands that corresponded to vector sequences. Although it hybridized to an apparent 3.1-kbp *Bgl*I fragment in pBW120, that band was less intense and probably resulted from incomplete digestion, which could produce such a fragment with vector sequences. That this is the case is supported by the lack of hybridization to bands produced with other enzymes that correspond to the 3.1-kbp *Bgl*I fragment of the *E. coli phn* region (Fig. 3 and 4). All other fragments of both C-P lyase plasmids that hybridized to the phosphonatase clone were accountable as fragments that contain vector sequences. pEG5166 includes *lacZ* DNA, *ori*, and *bla* sequences in common with pBW120, a pUC18-based plasmid.

We examined genomic DNAs of a $Pn^+$ *phn*(EcoB) and Δ(*phnC?DEFGHIJKLMNOP*)*33-30 E. coli* hosts and *E. aerogenes* by using pBW120, pEG5166-KL38, and pEG 5166-KL124 as probes. Our data showed that both C-P lyase clones hybridized strongly to several chromosomal DNA bands of the same sizes from $Pn^+$ *E. coli* and *E. aerogenes*. The hypothesis that these bands correspond to the *phnC*-to-*phnP* gene cluster in *E. coli* was verified by their absence in the Δ(*phnC?DEFGHIJKLMNOP*)*33-30* mutant. Also, the *E. aerogenes* C-P lyase plasmid failed to hybridize to any specific *E. coli* bands in the *phnCDE* region, which encodes the Pn transporter. Therefore, the *E. aerogenes* C-P lyase plasmid does not encode homologous genes for a Pn transporter, in full agreement with the complementation data above.

As expected, the sizes of the homologous chromosomal restriction fragments differed in *E. aerogenes* and *E. coli*. *E. aerogenes* DNAs had 4.9-, 3.4-, 1.6-, and 1.1-kbp *Eco*RV fragments, 8.5-, 3.3-, 2.5-, 1.6-, and 0.7-kbp *Sal*I fragments, and *Bgl*II and *Hin*dIII fragments greater than 10 kbp that hybridized to both *E. coli* and *E. aerogenes* C-P lyase plasmids, pBW120 and pEG5166-KL124, respectively. These restriction fragments probably correspond to the *E. aerogenes* locus for a C-P lyase. pBW120 hybridized also to additional *E. aerogenes* restriction fragments. However, in the absence of a detailed physical map of *E. aerogenes* genes for Pn metabolism, we could not ascertain whether such additional bands corresponded to *E. aerogenes* Pn transport genes or to other genes on pBW120 (data not shown).

The *E. aerogenes* phosphonatase plasmid also hybridized strongly to *E. coli* DNA, but no homologous fragment coincided with one(s) detected with either C-P lyase plasmid. Also, no fragment detected with the phosphonatase plasmid was absent in the *E. coli* Δ(*phnC?DEFGHIJKLM NOP*)*33-30* mutant. Our data showed that if the phosphonatase plasmid encodes a Pn transporter, then that Pn transport gene(s) is not homologous to the *E. coli phnCDE* region. These data further support the results above on the hybridization between plasmid DNAs. The homology to *E. coli* DNA detected with the phosphonatase plasmid was probably due to unrelated genes that happen to be linked to genes for the phosphonatase pathway in *E. aerogenes*. Importantly, no *E. aerogenes* chromosomal fragment detected with the phosphonatase plasmid coincided with one that was also detected with either C-P lyase plasmid. In other words, no *E. aerogenes* chromosomal restriction fragment includes genes encoding both a C-P lyase and a phosphonatase pathway (data not shown). Therefore, the chromosomal loci for these two pathways are unlinked.

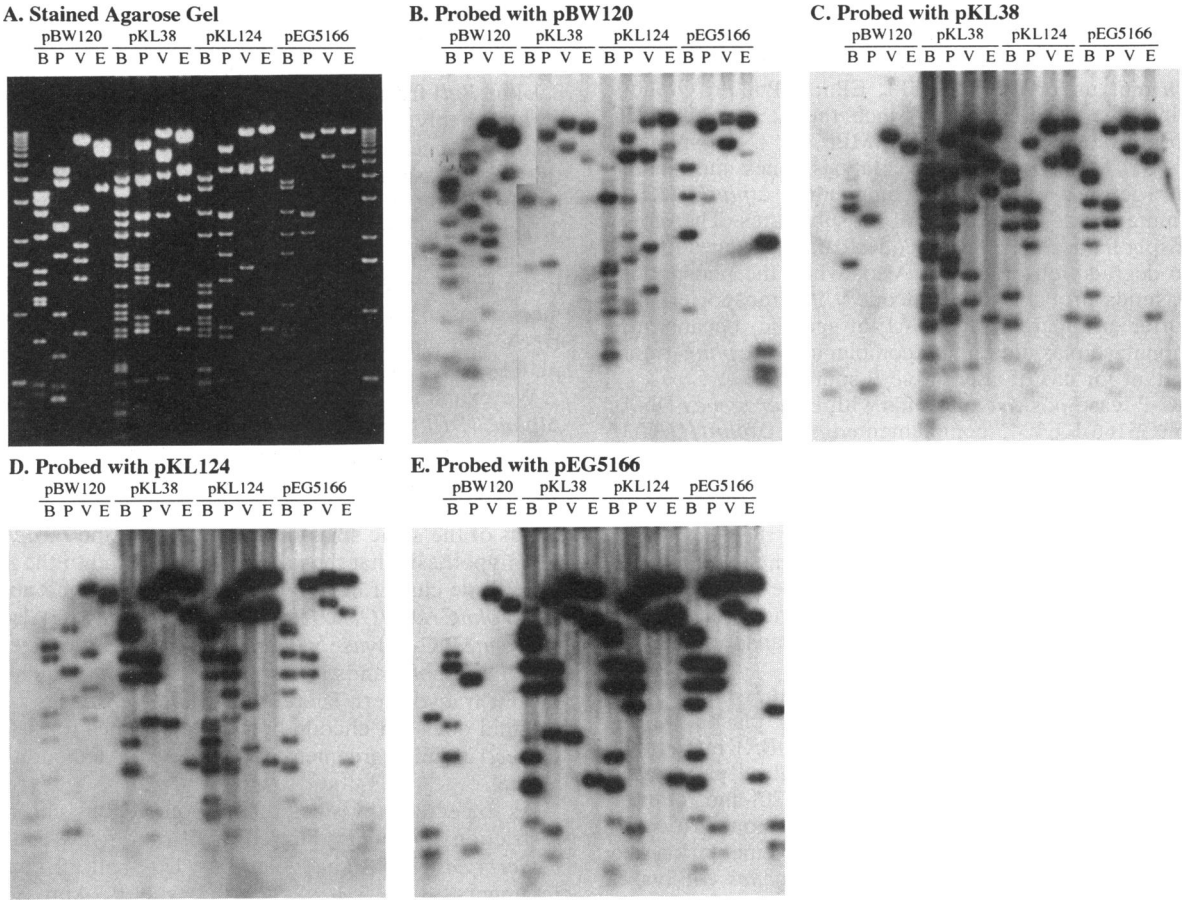


FIG. 4. Hybridization of *E. aerogenes* and *E. coli* C-P lyase genes. Plasmid DNAs were digested singly with *Bgl*I (B), *Pvu*II (P), *Eco*RV (V), or *Eco*RI (E), and one-half of each digest was separated by electrophoresis on duplicate 0.7% agarose gels that were run side-by-side. Each gel was blotted onto two membranes that were then probed and autoradiographed, as described elsewhere (19). (A) One of the duplicate stained agarose gels transferred to membranes. (B) Autoradiograph of a membrane probed with pBW120. This panel shows a composite of two exposures of the same membrane; the left portion was cut from an autoradiograph for a shorter exposure. (C) Autoradiograph of a membrane probed with pEG5166-KL38. (D) Autoradiograph of a membrane probed with pEG5166-KL124. (E) Autoradiograph of a membrane probed with the vector pEG5166. Lanes with pEG5166-KL38 and pEG5166-KL124 DNAs are labeled pKL38 and pKL124, respectively. Both outside lanes contain the 1-kbp ladder (GIBCO BRL, Bethesda, Md.) which includes 1.6-, 0.5-, and 0.4-kbp fragments that hybridize to vector DNA in pBW120 and to pEG5166. Since mini-Mu cloning results in the loss of some pEG5166 sequences, only the 0.5- and 0.4-kbp fragments of the 1-kbp ladder hybridize to pEG5166 mini-Mu clones. See Materials and Methods for further details.

## DISCUSSION

We isolated two kinds of complementing plasmids from *E. aerogenes* DNA libraries which probably encode different Pn degradative pathways. One kind encodes genes for a phosphonatase pathway; the other encodes genes for a C-P lyase pathway. The former allows use of only AEPn as a P source, while the latter allows use of AEPn, DMPt, EPn, MPn, and Pt. DNA hybridization of plasmid DNAs showed that the genes for the two pathways are not homologous; DNA hybridization of genomic DNAs showed that the genes for the two pathways are also not linked on the *E. aerogenes* chromosome. This is because no chromosomal restriction fragment from *E. aerogenes* hybridized to both kinds of plasmids, even though some fragments were quite large. That these plasmids carry different genes was shown both by the lack of homology between the phosphonatase and C-P lyase plasmids and between the phosphonatase plasmid and genes on the *E. coli* chromosome near and including the *phnC*-to-*phnP* gene cluster. In contrast, we verified that we had cloned genes for an *E. aerogenes* C-P lyase by the strong homology between genes for the *E. aerogenes* C-P lyase and catalysis genes of the *E. coli phnC*-to-*phnP* gene cluster. Therefore, genes encoding the *E. aerogenes* phosphonatase and C-P lyase pathways are unrelated, while the genes encoding the *E. aerogenes* and *E. coli* C-P lyase pathways are homologous.

The existence in *E. aerogenes* of two Pn degradative pathways explains why different physiological effects were seen during growth on AEPn, MPn, and Pt. *E. aerogenes* showed no diauxie effect during the shift from $P_i$ to AEPn utilization, while *E. coli* showed a distinct diauxie effect during the same shift. Apparently, *E. aerogenes* behaves differently during growth on AEPn, because it primarily uses the phosphonatase pathway to metabolize AEPn and uses the C-P lyase pathway to metabolize MPn and Pt. In contrast, *E. coli* degrades Pn solely via a C-P lyase pathway and therefore always showed a growth diauxie. *E. aerogenes* also behaved differently in that it made Bap at a reduced rate

during growth on AEPn and, like *E. coli*, made Bap at a high rate during steady growth on other Pn. Apparently, an excess of $P_i$ is produced by the phosphonatase pathway and the $P_i$ released leads to (partial) repression of the PHO regulon. This repression of the PHO regulon in *E. aerogenes* during growth on AEPn is reminiscent of the repression of the PHO regulon in *E. coli* during growth on phosphate esters (22).

Although we obtained no evidence for a Pn transport gene(s) on the *E. aerogenes* phosphonatase plasmid, it probably does encode a transporter. The transporter is apparently AEPn specific, however. It is likely that it encodes a transporter because the enzymes of the phosphonatase pathway are probably cytoplasmic and because the phosphonatase plasmid complements mutants with the *phnCDE* genes deleted and these genes encode the only AEPn transporter in *E. coli*. The genes encoding such an AEPn-specific transporter may simply not be homologous to the *phnCDE* genes for the *E. coli* Pn transporter. In addition, *B. cereus* has an apparent $P_i$-repressible, AEPn-specific transporter for its phosphonatase pathway (14). The enzymes in the phosphonatase pathway are also probably cytoplasmic, because the first step of AEPn degradation in *B. cereus* and *P. aeruginosa* involves a transaminase. The AEPn transaminase in *P. aeruginosa* A237 uses pyruvate as an amino group acceptor (3); it therefore probably requires pyridoxal phosphate, a cofactor found only in the cytoplasm. Biochemical data on enzymes of the apparent *E. aerogenes* phosphonatase pathway are lacking, however.

One reason we studied Pn degradation in *E. aerogenes* IFO 12010 is because it apparently used a broader spectrum of compounds as the sole P source than *E. coli*. *E. aerogenes* grows well on AEPn, DMPt, EPn, MPn, PnAc, and Pt; *E. coli* also grows reasonably well on AEPn, EPn, MPn, and Pt, poorly on PnAc, and very poorly or not at all on DMPt. Such growth differences between *E. aerogenes* and *E. coli* may reflect different specificities for Pn uptake or Pn degradation. Since plasmids that encode the *E. aerogenes* C-P lyase require an intact *E. coli* Pn transporter for complementation, we can suggest which effects are due to differences in Pn transport and which effects are due to differences in the C-P lyase. Plasmids with genes for the *E. aerogenes* C-P lyase allow *E. coli* to degrade DMPt. Thus, there is an apparent difference in the substrate specificity of the C-P lyase with respect to DMPt. On the contrary, the same plasmids do not allow *E. coli* to grow well on PnAc. Therefore, the poor growth of *E. coli* on PnAc may be due to poor uptake. Also, we isolated *E. coli* mutants that grow well on PnAc and showed that one such mutant has an alteration in a gene for the *E. coli phnCDE*-encoded Pn transporter (8; unpublished data).

Early studies showed that the phosphonatase of *B. cereus* was made during $P_i$ limitation (14). The *E. coli* PHO regulon encodes genes expressed during $P_i$ limitation for use of alternative P sources. Expression of these genes requires the transcriptional activator PhoB. Since an *E. aerogenes* phosphonatase plasmid failed to complement an *E. coli* Δ(*phoBR*) mutant, a gene(s) for the phosphonatase pathway is probably under PHO regulon control. Likewise, the *E. aerogenes* C-P lyase genes are probably also under PHO regulon control, since they are homologous to genes in *E. coli* (19).

In summary, our data show that *E. aerogenes* has two pathways for Pn degradation, a phosphonatase pathway and a C-P lyase pathway. Evidence for two pathways was based on differences in substrate specificity and the lack of DNA homology among the genes cloned into the two kinds of plasmids. Since genes for both the *E. aerogenes* phosphonatase and *E. coli* C-P lyase pathways are under PHO regulon control in *E. coli*, various members of the family *Enterobacteriaceae* probably use Pn as a common alternative P source in nature. Interestingly, we previously reported that *Salmonella typhimurium* differed from several other members of the family *Enterobacteriaceae* because *S. typhimurium* used only AEPn but no other Pn as the sole P source. In light of our evidence for a phosphonatase pathway in *E. aerogenes*, *S. typhimurium* probably has only a phosphonatase pathway. We do not know why such closely related bacteria should have genes for two fundamentally different Pn degradative pathways. It is likely related to the different niches that these bacteria occupy in nature. Our *E. coli phn* mutants with plasmids for the *E. aerogenes* phosphonatase pathway provide a model system for new studies on Pn metabolism.

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## REFERENCES

1. **Chen, C.-M., Q. Ye, Z. Zhu, B. L. Wanner, and C. T. Walsh.** 1990. Molecular biology of carbon-phosphorus bond cleavage: cloning and sequencing of the *phn* (*psiD*) genes involved in alkylphosphonate uptake and C-P lyase activity in *Escherichia coli* B. J. Biol. Chem. **265**:4461–4471.
2. **Cordaro, J. C., T. Melton, J. P. Stratis, M. Atagün, C. Gladding, P. E. Hartman, and S. Roseman.** 1976. Fosfomycin resistance: selection method for internal and extended deletions of the phosphoenolpyruvate:sugar phosphotransferase genes of *Salmonella typhimurium*. J. Bacteriol. **128**:785–793.
3. **Dumora, C., A.-M. Lacoste, and A. Cassaigne.** 1983. Purification and properties of 2-aminoethylphosphonate: pyruvate aminotransferase from *Pseudomonas aeruginosa*. Eur. J. Biochem. **133**:119–125.
4. **Groisman, E. A., and M. J. Casadaban.** 1986. Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J. Bacteriol. **168**:357–364.
5. **Groisman, E. A., and M. J. Casadaban.** 1987. Cloning of genes from members of the family *Enterobacteriaceae* with mini-Mu bacteriophage containing plasmid replicons. J. Bacteriol. **169**: 687–693.
6. **Kennedy, K. E., and G. A. Thompson.** 1970. Phosphonolipids: localization in surface membranes of *Tetrahymena*. Science **168**:989–991.
7. **Metcalf, W. W., P. M. Steed, and B. L. Wanner.** 1990. Identification of phosphate starvation-inducible genes in *Escherichia coli* K-12 by DNA sequence analysis of *psi*::*lacZ*(Mu dl) transcriptional fusions. J. Bacteriol. **172**:3191–3200.
8. **Metcalf, W. W., and B. L. Wanner.** 1991. Involvement of the *Escherichia coli phn* (*psiD*) gene cluster in assimilation of phosphorus in the form of phosphonates, phosphite, $P_i$ esters, and $P_i$. J. Bacteriol. **173**:587–600.
9. **Metcalf, W. W., and B. L. Wanner.** Unpublished data.
10. **Murata, K., N. Higaki, and A. Kimura.** 1989. A microbial carbon-phosphorus bond cleavage enzyme requires two protein components for activity. J. Bacteriol. **171**:4504–4506.
11. **Murooka, Y., N. Takizawa, and T. Harada.** 1981. Introduction of bacteriophage Mu into bacteria of various genera and intergeneric gene transfer by RP4::Mu. J. Bacteriol. **145**:358–368.
12. **Olsen, D. B., T. W. Hepburn, M. Moos, P. S. Mariano, and D. Dunaway-Mariano.** 1988. Investigation of the *Bacillus cereus* phosphonoacetaldehyde hydrolase. Evidence for a schiff base

mechanism and sequence analysis of an active-site peptide containing the catalytic lysine residine. Biochemistry **27**:2229–2234.

13. **Ron, E. Z.** 1975. Growth rate of *Enterobacteriaceae* at elevated temperatures: limitation by methionine. J. Bacteriol. **124**:243–246.
14. **Rosenberg, H., and J. M. La Nauze.** 1967. The metabolism of phosphonates by microorganisms. The transport of aminoethylphosphonic acid in *Bacillus cereus*. Biochim. Biophys. Acta **141**:79–90.
15. **Ross, W., S. H. Shore, and M. M. Howe.** 1986. Mutants of *Escherichia coli* defective for replicative transposition of bacteriophage Mu. J. Bacteriol. **167**:905–919.
16. **Wackett, L. P., S. L. Shames, C. P. Venditti, and C. T. Walsh.** 1987. Bacterial carbon-phosphorus lyase: products, rates, and regulation of phosphonic and phosphinic acid metabolism. J. Bacteriol. **169**:710–717.
17. **Wanner, B. L.** 1986. Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Mol. Biol. **191**:39–58.
18. **Wanner, B. L.** 1990. Phosphorus assimilation and its control of gene expression in *Escherichia coli*, p. 152–163. *In* G. Hauska and R. Thauer (ed.), The molecular basis of bacterial metabolism. Springer-Verlag, Heidelberg, Germany.
19. **Wanner, B. L., and J. A. Boline.** 1990. Mapping and molecular cloning of the *phn* (*psiD*) locus for phosphonate utilization in *Escherichia coli*. J. Bacteriol. **172**:1186–1196.
20. **Wanner, B. L., and B.-D. Chang.** 1987. The *phoBR* operon in *Escherichia coli* K-12. J. Bacteriol. **169**:5569–5574.
21. **Wanner, B. L., and R. McSharry.** 1982. Phosphate-controlled gene expression in *Escherichia coli* using Mud1-directed *lacZ* fusions. J. Mol. Biol. **158**:347–363.
22. **Willsky, G. R., and M. H. Malamy.** 1976. Control of the synthesis of alkaline phosphatase and the phosphate-binding protein in *Escherichia coli*. J. Bacteriol. **127**:595–609.