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Mapping and Molecular Cloning of the *phn* (*psiD*) Locus for Phosphonate Utilization in *Escherichia coli*

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The *Escherichia coli phn* (*psiD*) locus encodes genes for phosphonate (Pn) utilization, for *phn* (*psiD*) mutations abolish the ability to use as a sole P source a Pn with a substituted C-2 or unsubstituted hydrocarbon group such as 2-aminoethylphosphonate (AEPn) or methylphosphonate (MPn), respectively. Even though the *E. coli* K-12 phosphate starvation-inducible (*psi*) *phn* (*psiD*) gene(s) shows normal phosphate (P_i) control, Pn utilization is cryptic in *E. coli* K-12, as well as in several members of the *E. coli* reference (ECOR) collection which are closely related to K-12. For these bacteria, an activating mutation near the *phn* (*psiD*) gene is necessary for growth on a Pn as the sole P source. Most *E. coli* strains, including *E. coli* B, are naturally Phn$^+$; a few *E. coli* strains are Phn$^-$ and are deleted for *phn* DNA sequences. The Phn$^+$ *phn*(EcoB) DNA was molecularly cloned by using the mini-Mu in vivo cloning procedure and complementation of an *E. coli* K-12 Δ*phn* mutant. The *phn*(EcoB) DNA hybridized to overlapping λ clones in the *E. coli* K-12 gene library (Y. Kohara, K. Akiyama, and K. Isono, Cell 50:495–508, 1987) which contain the 93-min region, thus showing that the *phn* (*psiD*) locus was itself cloned and verifying our genetic data on its map location. The cryptic *phn*(EcoK) DNA has an additional 100 base pairs that is absent in the naturally Phn$^+$ *phn*(EcoB) sequence. However, no gross structural change was detected in independent Phn$^+$ *phn*(EcoK) mutants that have activating mutations near the *phn* locus.

The carbon-phosphorus (C-P) bond is chemically very stable. Indeed, early evidence for natural C-P compounds was based upon finding organophosphorus that was released during combustion which resisted strong-acid and strong-base hydrolysis (17). The first natural C-P compound was isolated from ciliates and identified as 2-aminoethylphosphonate (AEPn) in 1959 (16). AEPn is a structural analog of the ethanolamine P_i moiety in phospholipids. Lipids with C-P bonds (phosphonolipids), corresponding to the phosphonate (Pn) analogs of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine, exist in various organisms from protozoa to mammals. Pn also exist covalently bound to proteins as well as other structural components of the cell (15). The recent finding of AEPn in *Escherichia coli* during the development of genetic competence for DNA transformation (R. N. Reusch, E. A. Osuch, and N. R. Nirmala, Fed. Proc. **46**:2217, 1987) implies that Pn may have a fundamental role. However, their function in nature is unknown. Since their role is probably related to the extreme stability of the C-P bond, the biochemistry of C-P compounds is important. Additionally, various natural and synthetic Pn act as enzyme inhibitors; antibacterial, antifungal, or antiviral agents; or as herbicides (1, 10, 25, 35). Thus, both the biosynthesis and biodegradation of the C-P bond are of interest.

Cleavage of the C-P bond in AEPn is thought to proceed via a transamination-mediated dephosphonation in two steps: AEPn is first deaminated to phosphonoacetaldehyde, which is then hydrolyzed to P_i and acetaldehyde by phosphonoacetaldehyde phosphonohydrase (trivial name, phosphonatase). A phosphonatase that degrades AEPn was purified from *Bacillus cereus* and biochemically characterized in vitro (20). Although the hydrolysis of the C-P bond of phosphonoacetaldehyde may occur via the formation of an enzyme-bound imine with an amino group of the enzyme (19), a similar reaction pathway is not feasible for alkyl- or phenylphosphonates, since the products of their degradation are the corresponding hydrocarbons (8, 9) and P_i. Breaking the C-P bond in an alkylphosphonate such as methylphosphonate (MPn) apparently proceeds by an enzyme via a direct dephosphonation, for which the trivial name C-P lyase was adopted (36). The biochemical mechanism for C-P bond fission by a lyase is poorly understood, for it has been difficult to detect a C-P lyase activity in cell extracts. The first reports of a cell-free C-P lyase activity, from an *Enterobacter aerogenes* extract, have appeared only recently (26, 27).

Further progress in understanding the metabolism of C-P compounds could be greatly aided by molecular and genetic analyses of the genes involved. The observation that an in vivo C-P lyase activity increases during P_i limitation (36) suggested that a gene(s) for Pn utilization was a member of the phosphate, PHO, regulon (39). This was confirmed by showing that the *psiD* gene(s) was necessary for *E. coli* K-12 to use a Pn as the sole P source (36a). Accordingly, the *psiD* region was renamed the *phn* (phosphonate) locus in this study.

An activating mutation is necessary for *E. coli* K-12 to degrade a Pn. In this paper, we describe the cryptic *phn*(EcoK) allele and mutations that lead to its activation. We describe several *E. coli* strains that naturally differ in their Phn phenotype, the mapping and molecular cloning of the naturally Phn$^+$ *phn*(EcoB) locus, and evidence for a gross DNA difference between the cryptic *phn*(EcoK) and functional *phn*(EcoB) DNAs. The complete DNA sequence of the 15.6-kilobase-pair (kb) *phn*(EcoB) DNA insert of the Phn$^+$ complementing plasmid pBW120, which is described here, has now been determined (C.-M. Chen, Q. Ye, Z. Zhu, B. L. Wanner, and C. T. Walsh, J. Biol. Chem., in press).

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MATERIALS AND METHODS

Media, chemicals, and other materials. Most of the materials used in this work were reported previously (37, 42). MPn and AEPn were purchased as free acids from Sigma Chemical Co., St. Louis, Mo. Ethylphosphonic (EPn) acid was from Alfa, Danvers, Mass. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (XP) were from Bachem (Torrance, Calif.) and are the blue dyes for detecting β-galactosidase and bacterial alkaline phosphatase (Bap), respectively. NZ amine agar was used for making plaque-purified λ lysates by a standard plate overlay procedure. It contained (per liter) 10 g of casein hydrolysate (type I, enzymatic; Sigma), 2 g of $MgCl_2 \cdot 7H_2O$, 5 g of NaCl, and 1 mg of thiamine. The top agar was made with 0.4% agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) when the bacteriophage DNA was cleaved with endonucleases. Plasmid and F′ strains were grown on TYE agar with an appropriate antibiotic. Ampicillin, kanamycin, streptomycin, and tetracycline were present at 100, 50, 100, and 15 μg/ml, respectively. Tetracycline agar also contained 2.5 mM PP_i, except for Pn media. Glucose morpholinepropanesulfonic acid (MOPS) medium with 0.1 mM or 2 mM P_i was used to test for P_i-regulated gene expression. Phosphonic acids were used in MOPS medium at 0.05 mM in place of P_i. Media were solidified with 1.5% agar, using either Bacto-Agar or purified agar (Difco Laboratories, Detroit, Mich.). Purified agar was used to test for the use of various chemicals as a sole P source, in P1 transductions, in DNA transformations, and in some mutagenesis experiments for selecting growth on a Pn as the sole P source.

Molecular genetics. Hfr crosses, transductions with P1 *kc*, and Tn*10* mutagenesis with λNK561 were done as described earlier (37). Recombinants were always purified at least once nonselectively before final scoring for relevant phenotypes. P1 crosses were usually done between donor and recipient cells that differed for an unlinked marker (37). The Δ(*proC phoBR*)*9-6* mutation was introduced by cotransduction with a near *tsx-234*::Tn*10*, and then a Pro^- transductant was made Pro^+ with P1 grown on *phoB23* and *phoB513*(Am) mutants (40).

Mu dl mutants are somewhat unstable because Mu dl transposes at a high frequency (5). More stable derivatives were made in each of three ways. (i) Each *phn* (*psiD*)::*lacZ*(Mu dl) was crossed into a Mu-1 lysogen by P1 transduction with selection for Amp^r. No transposition events were noticed in P1 crosses when P1*kc* lysates were made on clonally purified Mu dl mutants at 30°C and more than 500 transductants of a Mu-1 lysogen were examined for their Phn and P_i-regulated Lac phenotypes. (ii) Each *phn* (*psiD*)::*lacZ*(Mu dl) was swapped to a λp1(*209*) substitution by selecting for temperature-resistant (Ts^+) cells from turbid plaques, as described previously (42). In such swaps, about 1 to 8 of 10 Ts^+ colonies are routinely Amp^s and λ immune and show the same Lac regulation as the parent, as expected for a true swap. (iii) Each *phn* (*psiD*)::*lacZ*(Mu dl) was also swapped for the transposition-defective Mu dl-1734 element by infection with a heat-induced lysate of MALII-1734, as described elsewhere (6). In such crosses, anywhere from 1 to 50% of the Kan^r transductants selected at 30°C are Amp^s and show the same Lac regulation as the parent.

Testing for Pn utilization. Bacteria are routinely tested for Pn utilization by streaking on glucose MOPS Pn agar. Plates are made with Bacto-Agar when testing for cryptic *phn* alleles or purified agar when testing for a Phn^+ or Phn^- character. The higher apparent mutation frequency for cells with a cryptic *phn* allele on Bacto-Agar versus purified agar allows for easier distinction of Phn^- cells that are mutatable to Phn^+ from ones that are not mutatable to Phn^+. Cells are also streaked on glucose MOPS agar with 2 and 0.1 mM P_i, then tested for Bap synthesis by dripping onto the colonies a solution of 0.4% *p*-nitrophenolphosphate in 1 M Tris hydrochloride (pH 8.0). Bacteria that turn yellow rapidly are scored as Bap^+. Suitable Phn^+, Phn^-, cryptic *phn*(EcoK), Bap^+, Bap^-, or Bap-constitutive cells are always compared on the same plate. When indicated, methane production was monitored by gas chromatography (L. P. Wackett, C. P. Venditti, and C. T. Walsh, personal communication), as described previously (36).

Bacteria, phages, and plasmids. Strains used are listed in Table 1 or described below. *E. coli* HP2, HP4, HP5, HP12, HP15, and HP18 are independent isolates from a Purdue Swiss mouse, guinea pig, and sheep (J. Huxley-McCune, M. S. thesis, Purdue University, West Lafayette, Ind., 1965) and were provided by F. C. Neidhardt. The *E. coli* B derivative NC3 was from a laboratory stock (41). Wild-type *E. coli* B was from H. E. Umbarger. Two sets of the 72 *E. coli* reference (ECOR) strains (33) were tested; one set was made available by H. E. Umbarger and the other was made available by R. Selander. Both sets were received as stab cultures, and phenotypic differences were noticed among individual clones from some stabs. Four stabs contained both prototrophic and auxotrophic clones, and three others had Bap-constitutive or Bap^- clones among ones showing normal P_i-regulated Bap synthesis. Prototrophic cells that showed P_i-repressible Bap synthesis were isolated from all stabs except ECOR29, ECOR52, and ECOR71; these were tested for Pn utilization. ECOR29 and ECOR52 require nicotinic acid for growth (M. Levinthal, personal communication), so their Phn character was tested in the presence of nicotinate. The auxotrophy in ECOR71 was not identified by standard auxanography testing and therefore could not be tested for Pn utilization.

The other gram-negative bacteria included *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter cloacae*, *Hafnia alvei*, *Klebsiella pneumoniae* MK1, *Proteus vulgaris*, *Serratia marcescens* SU1, and *Shigella sonnei* (from H. E. Umbarger) and *Salmonella tryphimurium* LT2 (from L. Csonka). The *E. coli* B reference strain NC3 is a restriction-negative B/r whose construction was described previously (41). The mini-Mu plasmid cloning vector pEG5005 in MC1040 (12) was from E. Groisman, and pUC18 was from A. Shauer. Strain 71.18 [F′ 128 *traD38* $lacI^{q1}Z\Delta M15$ *pro*(*BA*)$^+$/DE5(*proBA*)XIII *lac hsdR4 supE44 endA sbcB15*] was from C. Squires, and NK7379 (F′pOX38*zff*::Tn*10-11*(Tc^r, also called Δ*16*Δ*17* mini-Tn*10* in reference 45) was from N. Kleckner. The 476 miniset of λ chimeric phages for the *E. coli* K-12 chromosome (18) was kindly provided by both F. C. Neidhardt and Y. Kohara. The *recB recC* host CES200, from F. Stahl, was used to propagate the λ*spi* miniset phages for DNA isolation.

The $traD^+$ Tc^r episome F′ 128 $lacI^{q1}Z\Delta M15$ *zff*::Tn*10-11*(tet) was made by crossing the mini-Tn*10*Δ*16*Δ*17* on the F′ pOX38 in NK7379 onto the *traD* F′ 128 of strain 71.18 by P1 transduction. To do this, P1 grown on NK7379 was used to

TABLE 1. Bacterial strains used in this study

Strain	Genotype[a]	Pedigree[b]	Source or construction
AKK231 (CGSC 6772)	HfrC *relA1 spoT1 ilv-299 metB1 zjd-2231*::Tn*10 ampCp1*		I. Booth via B. Bachman
AKK241 (CGSC 6773)	HfrC *relA1 spoT1 ilv-299 metB1 zje-2241*::Tn*10 ampCp1*		I. Booth via B. Bachman
BD792 (CGSC 6159)	F$^-$ *phoM*(wt)	W1485	37
BW979	Δ*lac-169 proC* IN(*rrnD-rrnE*)*1 rpsL ilv-1 his-29*(Am) *trpA9605*(Am) *trpR tsx ara malT*	FE103 via BW453 (36)	Spontaneous Str^r
BW1470	Δ*lac-169 phoR68 phn-33* (*psiD*)::*lacZ* (Mu d1) *pho-510 aroB crp-72 rpsL267 thi*	XPh1a via BW853 (42)	Pro$^+$ with P1 grown on BW478 (43)
BW2930	Δ*lac-169* Δ*phn*(*psiD*)*33-30 pho-510 aroB crp-72 rpsL267 thi*	XPh1a via BW853	Like BW1470, then Ts$^+$ spontaneously
BW3781	Δ*lac-169 phn-33*(*psiD*)::*lacZ*[λp1(*209*)] Ts$^+$ *phoR68 pho-510 aroB crp-72 rpsL267 thi*	XPh1a via BW1470	Ts$^+$ Amp^s lysogen with λp1(*209*)
BW3831	Δ*lac-169 phoM*(wt) *rpsL*	BD792 via BW3414 (36)	Spontaneous Str^r
BW3912	Δ*lac-169 pho-510 thi*	XPh1a via BW1589	37
BW4714	Δ*lac-169 pho-510 hsdR514*	BD792 via BW3739	37
BW9301	λ *recA*$^+$/*recA1* Δ*lac-169 pho-510 thi*	XPh1a via BW6050	Lysogenized with λ *recA*$^+$ (38)
BW9352	F′ 128 *zff*::Tn*10-11*(Tet) *lacI*^{q1}*Z*Δ*M15 pro*(*BA*)$^+$/Δ*lac-169 proC* IN(*rrnD-rrnE*)*1 rpsL ilv-1 his-29*(Am) *trpA9605*(Am) *trpR tsx ara malT*	FE103 via BW979	See text
BW9761	Δ*lac-169 phn-33*(*psiD*)::*lacZ*[λp1(*209*)] Ts$^+$ *phoR68 pho-510 rpsL267 thi*	XPh1a via BW3781	Aro$^+$ with P1 grown on BD792
BW9763	Δ*lac-169 phn-33*(*psiD*)::*lacZ*[λp1(*209*)] Ts$^+$ *phoR68 pho-510 thi*	XPh1a via BW3781	Like BW9761 except Str^s
BW9766	Δ*lac-169* Δ*phn*(*psiD*)*33-30 pho-510 thi*	XPh1a via BW2930	Aro$^+$ with P1 grown on BW3912
BW9907	Δ*lac-169 zjd-2*::Tn*10 phn*$^+$(EcoK) *pho-510 thi*	XPh1a via BW3912	Tc^r MPn$^+$ with λNK561
BW9949	Δ*lac-169 zjd-1*::Tn*10 phn*(EcoK) *phoR68 pho-510 rpsL267 thi*	XPh1a via BW9761	Tc^r Lac$^-$ with P1 grown on BW3912 Tn*10* pool (see text)
BW10197	Δ*lac-169 zjd-1*::Tn*10* Δ*phn*(*psiD*)*33-30 pho-510 thi*	XPh4 via BW9766	Tc^r with P1 grown on BW9949
BW10724	λ *recA*$^+$/*recA*::*cat-aadA* Δ*lac-169 pho-510 thi*	XPh4 via BW9301	Cm^r with P1 grown on GW4214
BW10733	Δ*lac-169 zjd-1*::Tn*10* Δ*phn*(*psiD*)*33-30 pho-510 hsdR514*	BD792 via BW4714	Tc^r Phn$^-$ with P1 grown on BW10197
BW11228	*recA*::*cat-aadA* Δ*lac-169* Δ*phn*(*psiD*)*33-30 pho-510 thi*	XPh1a via BW9766	Cm^r with P1 grown on BW10724
BW11334	F′ 128::Tn*10-11*(Tet) *lacI*^{q1}*Z*Δ*M15*/*recA*::*cat-aadA* Δ*lac-169* Δ*phn*(*psiD*)*33-30 pho-510 thi*	XPh1a via BW11228	Tc^r exconjugant with BW9352
BW12268	DE3(*lac*)X74 *phn-33*(*psiD*)::*lacZ* (Mu d1-*1734*) *phoM*(wt) *arcA1655 fnr-1655*	MG1655 via BW11331	Kan^r with P1 grown on BW12058
BW13208	Δ*lac-169 phn-33*(*psiD*)::*lacZ* (Mu d1-*1734*) *phoU35 pho-510 thi*	XPh1a via BW6504	Kan^r with P1 grown on BW12058
DF1062 (CGSC 6696)	*araD139* Δ(*araA-leu*)*7697* DE3 (*lac*)X74 *galE15 galK16 relA1 rpsL150 spoT1 zjg-920*::Tn*10 hsdR2*	MC4100	D. Fraenkel via B. Bachman
EMG2 (CGSC 4401)	F$^+$ λ$^+$ *phoM*(wt)		B. Bachmann
GR401 (CGSC 6715)	*araD139* Δ*lac-169 flbB5301* Δ(*his-gnd*)*296 relA1 rpsL150 cycA30*::Tn*10 deoC1*		I. R. Booth via B. Bachman
GW4214[c]	*recA*::*cat-aadA ara-14 galK2 lacY1 argE3 his-4 leu-6 proA2 thr-1 tsx-33 rpsL31 supE37 recB21 recC22 sbcB15 thi*	AB1157 via JC7623	46; G. C. Walker
MA156 (CGSC 6881)	Δ(*lacA-lacI*)*265 rpsL200* Δ(*hflA150*) *zje-599*::Tn*10* IN(*rrnD-rrnE*)*1*		M. A. Hoyt via B. Bachmann
MC1000	*araD139* Δ(*ara-leu*)*7679* DE3(*lac*)X74 *pho-510 galU galK rpsL thi*		6
MC4100	Δ*lac-169 phoM*(wt) *rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25 thi*(?)	MC4100	6
MG1655[d]	F$^-$ *phoM*(wt) *arcA1655 fnr-1655*	CW1485	M. Casadaban
SE3001	Δ*lac-169* Δ*malB1 rpsL150 relA1 araD139 thi*(?) *flbB5301 deoC1 ptsF25*	MC4100	36
W1485 (CGSC 5024)	F$^+$ *phoM*(wt)		B. Bachmann
			36
W3110	IN(*rrnD-rrnE*)*1 phoM*(wt)		42
Ymel (CGSC 503)	F$^+$ *phoM*(wt) *mel-1 supF58*		B. Bachmann

[a] *phoM*(wt) denotes the wild-type *phoM* operon because many common laboratory strains have the *pho-510* mutant form (44). Ts$^+$ means temperature-resistant growth was selected.

[b] All strains are descendents of *E. coli* K-12.

[c] We found that the *recA*::*cat* mutation in GW4214 (46) causes cells to be spectinomycin and streptomycin resistant, since the *cat* (*cam*) fragment that was used to construct this mutation also includes the *aadA* gene (34).

[d] This MG1655 has both *fnr* (B. Bachmann, personal communication) and *arcA* (M. R. Wilmes and B. L. Wanner, unpublished data) mutations.

infect 71.18. Tc^r transductants were selected, pooled, and mated with BW979, and Str^r and Tc^r doubly resistant exconjugants were selected. Strain BW9352 was saved as one that gave blue plaques on X-Gal agar with *lacZ*α donor M13mp phages (48) and transferred the Tc^r marked F′ 128 *lacI*^{q1}*ZΔM15* episome with a high efficiency, with counterselection for prototrophy.

Molecular cloning of the *phn*(EcoB) gene(s) for MPn utilization. The mini-Mu cloning technique (11) was used for molecular cloning of the *phn*(EcoB) gene(s). To do this, BW10748 [*phn*(EcoB) *hsdR514 pho-510*] was lysogenized with Mu *c62*(Ts$^-$) and then transformed to Amp^r and Kan^r with pEG5005 DNA. Several random transducing particle libraries were made by heat induction and used to infect the *recA*$^+$ Mu-1 lysogen BW10799 [*zjd-1*::Tn*10* Δ*phn* (*psiD*)*33-30*]. Kan^r transductants were selected on TYE-kanamycin agar and replica plated onto MPn agar. About 1% grew on the replicas.

Molecular biology procedures. Molecular biology procedures were described previously (38). Briefly, plasmid DNAs were isolated by a modified rapid alkaline procedure and phage DNAs were isolated from DNase I-treated lysates. Both phage and bacterial DNAs were purified by multiple phenol and phenol-chloroform-isoamyl alcohol extractions. DNA hybridizations were done with randomly primed [α-^{32}P]dCTP-labeled plasmid DNA with GeneScreen (Du Pont Co., Wilmington, Del.).

RESULTS

Pn utilization as a cryptic function in *E. coli* K-12. Earlier studies showed that unadapted *E. coli* K-12 grew only after a 60-h lag with MPn as the sole P source, but strains that had previously been adapted on MPn medium failed to show this extended lag phase (36a). This suggested that a mutation was responsible for the adaptation. To prove this, L-broth-grown (unadapted) wild-type bacteria were plated onto glucose MOPS Pn agar with MPn (or AEPn) as the sole P source, plus other required nutrients. *E. coli* K-12 strains BD792, BW3912, BW4714, EMG2 (wild-type K-12), MC1000, MC4100, MG1655, W1485, and W3110 each gave rise to Phn$^+$ colonies at an apparent frequency near 10^{-4}. Numerous representatives were purified nonselectively on TYE agar and then tested by replating on Pn agar. As all cells now formed Phn$^+$ colonies, a mutation is necessary for acquisition of a Phn$^+$ phenotype in *E. coli* K-12. Twelve mutants of BW3912 that were selected for growth on MPn and AEPn agars simultaneously acquired the ability to use either Pn. The high apparent mutation frequency (10^{-4} to 10^{-5}) was probably due to contaminating P sources, including P_i, which allowed for some growth on Pn agar prior to selection. When similar experiments were carried out with washed cells, no Phn$^+$ mutants were seen even when more than 10^9 bacteria were plated. Our inability to select Phn$^+$ mutants among a population of washed cells is not understood. It could be related to the apparent instability of the C-P lyase activity because (preselected) Phn$^+$ mutants also exhibited a low plating efficiency on Pn agar after simple washing (B. L. Wanner, D. Stark, and J. Boline, unpublished data).

Pn utilization in other *E. coli* and gram-negative bacteria. The above results show that Pn utilization is cryptic in *E. coli* K-12. This was unexpected, for several *E. coli* reportedly could use a variety of phosphonates, including MPn and AEPn, as the sole P source. However, earlier studies generally used *E. coli* Crooke's (14, 49), *E. coli* B (14), and others (24), but not *E. coli* K-12. The sole study that we are aware of with *E. coli* K-12 used cells that were adapted for growth on EPn (2); the same bacterial stocks also show a cryptic Phn phenotype (W. W. Metcalf and B. L. Wanner, unpublished data). As earlier studies were concerned primarily with the products of Pn metabolism, heavily inoculated broth cultures were often used and grown for extended periods (36 to 96 h). Therefore, Phn$^+$ mutants could have been unknowingly selected.

We asked whether any *E. coli* are naturally Phn$^+$ and whether various *E. coli* differ with respect to their Phn phenotype. When *E. coli* and its *hsdR* B/r derivative NC3 (41) were tested, both grew without a prolonged lag on either MPn or AEPn as the sole P source; i.e., they are naturally Phn$^+$. Also, *E. coli* NC3 made methane during growth on MPn, thus showing that NC3, like adapted K-12 bacteria, contains a C-P lyase activity. These data show that common laboratory *E. coli* differ in their ability to metabolize MPn or AEPn. Whereas the gene(s) for Pn utilization is cryptic in *E. coli* K-12, a similar gene(s) is functional in *E. coli* B.

Since *E. coli* K-12 and B were maintained in the laboratory for many years (3, 23), we tested other *E. coli* and related gram-negative bacteria for their natural ability to use a Pn as the sole P source. In general, cells showed the same phenotype with respect to the use of AEPn, MPn, or EPn. Three Phn phenotypes were uncovered among 80 independent *E. coli* isolates. Fourteen, including *E. coli* K-12, are naturally Phn$^-$ and are readily mutable to use a Pn as the sole P source. These 14 probably have a cryptic *phn* gene(s). Fifty-four, including *E. coli* B, 46 ECOR, and 7 HP strains, are naturally Phn$^+$ and therefore have a functional gene(s) for Pn utilization. Also, *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Serratia* spp. are Phn$^+$. Twelve *E. coli* in the ECOR collection are Phn$^-$ and failed to yield mutants that grew on MPn agar. These apparently have a nonfunctional (or perhaps deleted) gene(s) for Pn utilization. In addition to the three general classes, one Phn$^+$ ECOR and *S. typhimurium* differed in that they could use AEPn but neither alkyl Pn as the sole P source. The Phn character in *Edwardsiella*, *Proteus*, and *Shigella* spp. and *E. coli* ECOR71 is uncertain, since samples of these bacteria failed to grow sufficiently well, even on glucose MOPS P_i agar, to allow definitive characterization of their Phn character by plate tests.

The ECORs belong to six major phylogenetic groups that are designated by letters (33). Group A has 25 members, and a subgroup of 10 members within this group is similar to *E. coli* K-12. Of the 13 cryptic ECORs, 9 are members of the subgroup that is most similar to K-12; the 10th member of this subgroup ECOR2 is Phn$^-$. Two other cryptic clones, ECOR19 and ECOR24, are in the major group, group A, whereas the cryptic clones ECOR42 and ECOR43 are in group B1. Group A has 11 members that are cryptic, 3 that are Phn$^+$ (ECOR15, ECOR16, and ECOR22), and 11 others that are Phn$^-$. Of the 12 Phn$^-$ ECORs, ECOR37 is the sole Phn$^-$ strain that is not in group A. However, ECOR37 differs so much from the other ECORs that it is the sole member of group E. The 46 Phn$^+$ ECORs represent five of the major phylogenetic groups (A, B1, B2, C, and D), thus showing that the majority of *E. coli* are probably Phn$^+$. In addition, 22 of 25 group A members have either a cryptic or Phn$^-$ phenotype. Of the 46 others, 43 are Phn$^+$, 2 are cryptic, and 1 is Phn$^-$. We conclude that there exist three distinct Phn phenotypes in *E. coli* in nature and that ECORs displaying a particular Phn phenotype appear to be genetically related.

Use of Tn*10* to show that the cryptic *phn*(EcoK), Phn$^+$ *phn*(EcoB), and Phn$^+$-activating mutations are linked. Two mutants with a Tn*10* near the *phn* gene(s) proved useful in

characterizing various *phn* alleles. One mutant (*zjd-1*::Tn*10*) was isolated as having a Tn*10* near a *phn* (*psiD*)::*lacZ* fusion and was used in genetic crosses to show that the cryptic *phn*(EcoK) and Phn$^+$ *phn*(EcoB) alleles are linked. The other (*zjd-2*::Tn*10*) was found in an attempt to isolate a Tn*10*-induced mutation that activated the *phn*(EcoK) allele. Although the Phn$^+$ character in the original *zjd-2*::Tn*10* mutant proved to be due to a separate event, for the mutant had a Tn*10* near an activated Phn$^+$ *phn*(EcoK) allele, the *zjd-2*::Tn*10* was useful in showing that activating mutations lie near the cryptic *phn*(EcoK) locus, after the *zjd-2*::Tn*10* mutation was crossed into an appropriate background.

BW9761 {*phn-33*(*psiD*)::*lacZ*[λp1(*209*)] *phoR*} was used to place a Tn*10* near the *phn* locus. BW9761 expresses both the *phn*(*psiD*)::*lacZ* fusion and the *phoA* gene constitutively and is therefore Lac$^+$ and Bap$^+$ on indicator media in the presence of excess P_i. It is Phn$^-$ due to the *phn-33* (*psiD*)::*lacZ*[λp1(*209*)] mutation. To find a Tn*10* near the *phn* locus, BW9761 was infected with P1 grown on a pool of BW3912 that had random Tn*10*s, and Tc^r transductants were selected on TYE-tetracycline-X-Gal agar. Of 73 Tc^r transductants, 1 was white (Lac$^-$), λ^s, and Bap constitutive and readily mutated to Phn$^+$, which was expected for a transductant that received the cryptic *phn*(EcoK) allele from BW3912 along with a near Tn*10*. When P1 was grown on this mutant, BW9949 [*zjd-1*::Tn*10* *phn*(EcoK) *phoR*], and used to infect BW9763 {*phn-33*(*psiD*)::*lacZ*[λp1(*209*)] *phoR*}, 48 of 134 Tc^r transductant were Lac$^-$ and λ^s, and readily mutated to Phn$^+$. Therefore, *zjd-1*::Tn*10* is 37% linked to the *phn* mutation in BW9761.

The *zjd-1*::Tn*10* was crossed into a Δ*phn* mutant to aid in transferring the Δ*phn* mutation. A Δ*phn* host was used in constructing bacteria with various *phn* alleles, to ensure that no crossover could occur within *phn* DNAs that differ. The Δ*phn* (*psiD*)*33-30* deletion arose by excision of Mu d1 in BW853 [*phn-33* (*psiD*)::*lacZ*(Mu d1)], which was selected as a Ts$^+$ mutant that had lost all Mu d1 markers. BW9766 is a Δ*phn* descendent of BW853, whose construction is outlined in Table 1. When BW9766 was made Tc^r with P1 grown on a *zjd-1*::Tn*10* *phn*(EcoK) mutant, 75 of 136 transductants simultaneously acquired the cryptic *phn*(EcoK) phenotype while the others remained Phn$^-$. Thus, the *zjd-1*::Tn*10* is about 55% linked to the *phn*(EcoK) allele.

The *E. coli* B strain NC3 was used to show that the cryptic *phn*(EcoK) and Phn$^+$ *phn*(EcoB) alleles are linked. When NC3 was made Tc^r with P1 grown on a strain containing *zjd-1*::Tn*10* near *phn*(EcoK), about 60% were no longer Phn$^+$ but instead exhibited the cryptic *phn*(EcoK) character, for they now readily mutated to Phn$^+$. A similar proportion of NC3 transductants became Phn$^-$ when BW10197 [*zjd-1*::Tn*10* near Δ*phn* (*psiD*)*33-30*] was the donor. Although these data show that the *phn*(EcoK) and *phn*(EcoB) alleles differ, the Phn$^+$ character of NC3 could have still been due to an unlinked suppressor that is specific for the *phn*(EcoB) allele. This possibility was ruled out by crossing the *phn*(EcoB) allele into the *hsdR E. coli* K-12 derivative BW4714, in two steps. First, BW4714 was infected with P1 grown on Bw10197 to make the Tc^r Phn$^-$ transductant BW10733 [*zjd-1*::Tn*10* Δ*phn*(*psiD*)*33-30 hsdR514*], which was subsequently infected with P1 grown on NC3 to select Mpn$^+$ transductants. That 16 of 20 Phn$^+$ transductants simultaneously became Tc^s showed that the *phn* region was transferred. Therefore, the Phn$^+$ *phn*(EcoB) allele leads to a Phn$^+$ phenotype when it crossed into *E. coli* K-12. This two-step procedure for transferring the Phn$^+$ *phn*(EcoB) allele was useful in other experiments, including ones for the molecular cloning of the *phn*(EcoB) DNA, as described below.

TABLE 2. P1 linkage of the *phn* (*psiD*) locus with various Tn*10*s near the 93-min region[a]

Donor	Tn*10* marker	% Linkage[b]
AK231	*zje-2231*	10 (2/10)
AK241	*zje-2241*	100 (96/96)
DF1062	*zje-920*	7.5 (3/40)
GR401	*cycA30*	10 (1/10)
MA156	*zye-594*	7 (2/30)

[a] BW9761 {*phn-33*(*psiD*)::*lacZ*[λp1(*209*)] *phoR*} was infected with P1 grown on each donor, and Tc^r transductants were selected and examined for their Lac phenotype on TYE-tratrcycline-X-Gal agar.

[b] The percent linkage is based on the number of Lac$^-$ transductants as a fraction of the total transductants examined (shown in parentheses).

An attempt was made to isolate Tn*10*-induced Phn$^+$ mutants. Although none was identified, one Tn*10* was found which proved useful in showing that several independent activating mutations that cause a Phn$^+$ phenotype were linked to the *phn* locus. To do this, BW3912 [*phn*(EcoK)] was infected with ΔNK561 and plated onto glucose MOPS tetracycline-MPn agar, in four separate mutagenesis experiments. After nonselective purification of 28 apparent Tc^r Phn$^+$ mutants, 27 were Tc^r but only 4, from three independent selections, were Phn$^+$. When a P1 lysate made on each Phn$^+$ mutant was used to infect BW3831 [*phn*(EcoK)], to test for linkage of the Tn*10* and Phn$^+$ characters, about 50% (10 of 20) of the Tc^r transductants were Phn$^+$ for one donor, BW9907 [*zjd-2*::Tn*10* *phn*$^+$(EcoK)]; none was Phn$^+$ for the three other donors. Subsequent crosses confirmed that the *zjd-2*::Tn*10* was linked to but clearly separable from the activated Phn$^+$ *phn* allele in BW9907. We conclude that the Tn*10* and Phn$^+$ mutations occurred as a double event, which was selectable owing to the high apparent frequency of Phn$^+$ mutations in bacteria with the cryptic *phn*(EcoK) allele. The *zjd-2*::Tn*10* was subsequently used to show that 12 independently selected Phn$^+$ mutations that activated the cryptic *phn*(EcoK) allele could be cotransferred with the *zjd-2*::Tn*10* in P1 crosses (data not shown). Therefore, mutations near the *phn* locus readily occur which lead to activation of the cryptic *phn*(EcoK) allele.

Genetic mapping of the *phn* (*psiD*) locus. Earlier studies (42) showed that the *phn-31*, *phn-33*, and *phn-41* (*psiD*):: *lacZ*(Mu d1) fusions lie within the 90- to 2-min interval, between Hfr PO48 and PO120 of the Ra-2 and P801 strains, respectively. We therefore tested for P1 linkage between the *phn* (*psiD*)::*lacZ* fusions and markers in this region. Five Tn*10*s in the 92- to 93-min interval proved to be linked to the *phn* (*psiD*) locus, whereas other Tn*10*s in the 90- to 2-min interval region gave only Lac$^+$ transductants (Table 2 and data not shown). Since all Lac$^-$ transductants in Table 3 simultaneously lost λ immunity and regained the cryptic *phn*(EcoK) character, the *phn* locus lies near 93 min. The *zje-2241*::Tn*10* in AK241 proved especially useful in further characterization of the 93-min region.

The above results show that *zje-2241*::Tn*10* lies very near the *phn-33* (*psiD*)::*lacZ* fusion in BW9761. However, when the same donor lysate was used to infect the *phn-31*, *phn-33*, and *phn-41* (*psiD*)::*lacZ*(Mu d1) and Mu d1-1734 mutants, only 47% (147 of 313) of the Tc^r transductants became Lac$^-$ and drug sensitive and acquired the cryptic *phn*(EcoK) character, in each of the six crosses (data not shown). We suspect that greater linkage was seen when BW9761 {*phn-33* (*psiD*)::*lacZ*[λp1(*209*)]} was a recipient because BW9761 is

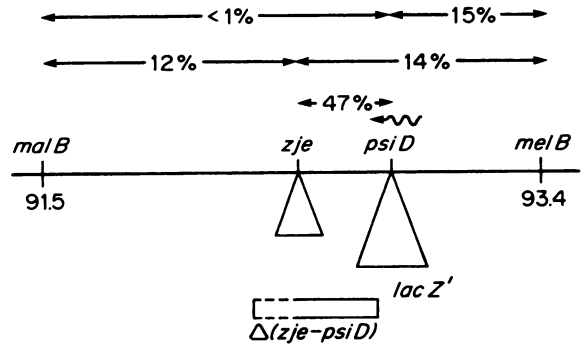

FIG. 1. Genetic markers near *phn* (*psiD*) locus. The *malB* and *mel* operons are those of Bachmann (3). The locations of the *zje-2241*::Tn*10* and *phn* (*psiD*)::*lacZ*(Mu d1) are described in the text. The open bar marked Δ(*zje-psiD*) shows the region that is thought to be deleted in BW9763, as described in the text. The wavy arrow shows the direction of transcription for the *phn* (*psiD*) gene. The percent (%) linkage between two markers is indicated by the numerals within the horizontal arrows near the top.

deleted for DNA downstream of the *phn-33* (*psiD*)::*lacZ* fusion, where the *zje-2241*::Tn*10* lies (Fig. 1 and data not shown).

P1 was grown on a *zje-2241*::Tn*10 phn-31* (*psiD*)::*lacZ*(Mu d1-1734) transductant described above and used to infect strain YMel (*mel-1*) to test for linkage of the *zje*::Tn*10* and *phn* (*psiD*)::*lacZ* fusion with other markers in the 93-min region. The *zje-2241*::Tn*10* and *phn-31* (*psiD*)::*lacZ*(Mu d1-1734) are about 14% linked to the *mel-1* marker with the gene order *zje-2241 phn-31* (*psiD*) *mel-1* (Table 3). In this cross, the *zje-2241*::Tn*10* and *phn-31* (*psiD*)::*lacZ*(Mu d1-1734) markers were about 94% linked. The reason for increased linkage in this cross is unknown. The four-factor cross with the *malB1* mutation implies the clockwise order *malB zje-2241 phn-31* (*psiD*) *mel-1* (Fig. 1). A counterclockwise orientation for the *phn* (*psiD*) promoter is inferred because we were unable to obtain recombinants between the *phn-33* (*psiD*)::*lacZ* fusion in BW9761, which we believe is deleted for DNA downstream of the fusion, and *zje-2241*::Tn*10* (Table 2 and data not shown).

Requirement for the *phn* (*psiD*) gene for Pn utilization. The *phn* (*psiD*)::*lacZ*(Mu d1) were isolated in a cryptic *phn* (EcoK) host as transcriptional fusions to promoters that show P_i-regulated *lacZ* expression (43). Later on, Wackett et al. (36a) discovered that the *phn* (*psiD*) mutants were unable to adapt and grow on MPn as the sole P source. To gain an insight into the nature of the activating mutations, each *phn* (*psiD*)::*lacZ* fusion was crossed into both cryptic (Phn^-) and activated (Phn^+) mutants and the transductants were tested for their Phn and Lac phenotypes. All transductants simultaneously became Phn^-; none showed an altered expression of the *lacZ* reporter gene. Even though the Phn^+-activating mutations lie near the *phn* locus, it seems unlikely that recombinants which showed altered Lac expression would have gone unnoticed, for in each case hundreds of transductants were tested (data not shown). These data therefore suggest that the activating mutations do not alter expression of the *phn* (*psiD*) promoter.

TABLE 3. P1 cotransduction in the *malB phn* (*psiD*) *mel* region[a]

Cross no.	No.
1.	
Psi-LacZ$^+$ Mel$^-$	88
Psi-LacZ$^+$ Mel$^+$	14
phn(EcoK) Mel$^-$	5
phn(EcoK) Mel$^+$	1
2.	
Mal$^+$ *phn*(EcoK) Mel$^+$	8
Mal$^-$ Psi-LacZ$^+$ Mel$^+$	28
Mal$^-$ Psi-LacZ$^+$ Mel$^-$	8
Mal$^-$ *phn*(EcoK) Mel$^+$	24

[a] Tc^r transductants were selected. In cross 1, the donor was *phn* (*psiD*)::*lacZ*(Mu d1-1734) *zje-2241*::Tn*10* and the recipient was YmeI (*mel-1*). A *phn*(*psiD*)::*lacZ*(Mu d1-1734) *zje-2241*::Tn*10 mel-1* transductant from cross 1 was used as the donor in cross 2, with SE3001 (Δ*malB1*) as the recipient. All Psi-LacZ$^+$ transductants were Kan^r and Phn^-, whereas the *phn*(EcoK) transductants readily mutated to Phn^+.

TABLE 4. Bap and β-galactosidase synthesis in *phn* (*psiD*)::*lacZ* fusion strains

Genotype[a]	Medium	Growth phase[b]	Bap sp act[c] (U/OD_{420})	β-Galactosidase sp act (U/OD_{420})
Wild type	0.1 mM P_i	Log	4.0	5.5
	0.1 mM P_i	Stationary	86.0	240
phoR	2.0 mM P_i	Log	88.1	109
phoU	2.0 mM P_i	Log	360	740

[a] BW12268, BW9763, and BW13208 were grown and assayed in glucose MOPS medium with 0.1 or 2.0 mM P_i.

[b] Several samples were taken for assay at hourly intervals throughout the log phase and during P_i limitation.

[c] Bap and β-galactosidase activities were measured in chloroform-sodium dodecyl sulfate-lysed cells as described previously (37). Units are nanomoles of product made per minute. OD_{420}, Optical density at 420 nm.

Pn utilization, *phn*(*psiD*)::*lacZ* fusion expression, and the PHO regulon. The *phn*(EcoK) promoter is genetically regulated much like the *phoA* gene (42). Also, MPn utilization, as determined by the amount of methane made, is similarly affected in various PHO regulon mutants (36a). In wild-type cells, both *phoA* and *phn* (*psiD*)::*lacZ* expression is induced about 40-fold upon P_i limitation (Table 4). Expression of both the *phoA* gene and *phn* (*psiD*)::*lacZ* fusion is rendered partially constitutive in an XPh *phoR* mutant and fully constitutive in a nearly isogenic *phoU* mutant. These data corroborate earlier evidence that the *phoA* and *phn* (*psiD*) promoters are coregulated as members of the PHO regulon.

The data on *phn* (*psiD*)::*lacZ* expression were obtained in cells with the cryptic *phn*(EcoK) allele (Table 4). Also, methane production was measured in unadapted K-12 cells, which, however, were grown sufficiently to allow for an adaptation (36a). It is conceivable that the activated form of the cryptic *phn*(EcoK) and the naturally Phn^+ *phn*(EcoB) allele are regulated differently. To test this, we crossed the Δ(*proC phoBR*)*9-6*, *phoB23*, and *phoB513*(Am) mutations (40) into the Phn^+ *phn*(EcoK) mutant BW9752 and the naturally Phn^+ *phn*(EcoB) strain NC3. Since PhoB is a transcriptional activator of the PHO regulon (39, 42), *phoB* mutations abolish the expression of several *phoB*-dependent promoters, including *phn* (*psiD*). Each *phoB* mutation abolished the ability of BW9752 or NC3 to grow on a Pn as the sole P source. We conclude that both activated Phn^+ *phn* (EcoK) and naturally Phn^+ *phn*(EcoB) alleles, like the *phn* [*psiD*(EcoK)]::*lacZ* fusions, require PhoB for expression.

Molecular cloning of the *phn*(EcoB) DNA. The Phn^+ *phn* (EcoB) gene(s) was cloned with the mini-Mu vector pEG5005 (11), as described in Materials and Methods. Thirty-seven Phn^+ clones were chosen from TYE-kanamycin master plates and characterized. Their plasmid DNAs were isolated, examined by restriction endonuclease diges-

tion, and tested for their *phn* character by transforming the *recA* Mu-1 lysogen BW11477 [Δ*phn* (*psiD*)*33-30 recA*::*cat-aadA*]. When the Phn$^+$ transformants were grown on MPn, all made methane. Since the amount of methane made was always linearly dependent upon the growth yield, none appeared to overproduce a C-P lyase activity (data not shown).

More than 20 Phn$^+$ plasmids contained a 5.3-kb *Hin*dIII fragment corresponding to the pEG5005 backbone plus two larger *Hin*dIII fragments, one between 5.4 and 12 kb and another 18 kb or larger. There were also 1.2-, 3.5-, and 9-kb *Eco*RI fragments in common plus one or two additional fragments, which totaled about 18 kb. The 1.2-kb *Eco*RI fragment in common is from the vector pEG5005 (11). Our finding of common-sized 3.5- and 9-kb *Eco*RI fragments in all Phn$^+$-complementing plasmids implied that each had DNA from the same chromosomal region. Four plasmids, including pBW101 and pBW112, were studied further. Several subclones were made that gave Phn$^+$ transformants of a RecA$^+$ *phn* mutant but not of a *recA phn* mutant. The Phn$^+$ transformants of the RecA$^+$ host apparently arose by marker rescue.

The plasmid pBW112 had two *Bam*HI sites, one in the chromosomal insert and one in the vector. The 15.6-kb *Bam*HI fragment from pBW112 was inserted into the *Bam*HI site in pUC18 to make the Phn$^+$-complementing plasmid pBW120. The removal of 4 kb from one end or 3 kb from the other gave plasmids that no longer complemented. The complete DNA sequence for the 15.6-kb fragment in pBW120 shows 17 predicted open reading frames (ORFs) in a possible *phn* operon structure, which we arbitrarily designated, in alphabetical order, as *phnA* through *phnQ* (Chen et al., in press). Our largest plasmids that no longer complement have (i) a deletion that removes the chromosomal DNA of the insert which is upstream of the proposed *phn* operon to within the *phnC* ORF or (ii) a deletion that removes the downstream DNA from within and beyond the *phnM* ORF (unpublished results from our laboratory). Therefore, DNA sequences for the *phnC* through *phnM* Orfs are at least necessary for complementation.

Physical mapping of the *phn*(EcoB) clone with an *E. coli* K-12 λ gene library. Our genetic data show that the *phn* locus lies between the *malB* and *mel* operons. To show that the *phn* region was molecularly cloned and to verify the mapping, we tested hydridization with phage DNAs from an *E. coli* K-12 λ gene library (18). Four adjacent λ clones from the 93-min region show DNA homology with pBW120 (Fig. 2). We conclude that the *phn* (*psiD*) region was cloned by complementation for Pn utilization and that the mapping data are accurate.

Figure 3 shows a restriction map for the sequenced *phn* DNA (Chen et al., in press) for nine enzymes that we used to examine homologous chromosomal DNAs. The enzymes included all eight enzymes used to map the *E. coli* chromosome, plus *Bgl*II. The map is oriented counterclockwise, to show a rightward (counterclockwise) direction for *phn* (*psiD*) transcription. Overall, our restriction map agrees reasonably well with the *E. coli* K-12 restriction map for the 93-min region, with a few minor exceptions. Accordingly, the *phn* sequences cloned probably correspond to a 15.6-kb portion of a 30.5-kb *Bam*HI fragment from kb 4380 to 4410 on the *E. coli* map (18). The chromosomal *Bam*HI site in Fig. 3 would therefore correspond to the BamHI site near kb 4410. The rightmost 114 base pairs and vector *Bam*HI site are, as expected, from the mini-Mu vector pEG5005 (11),

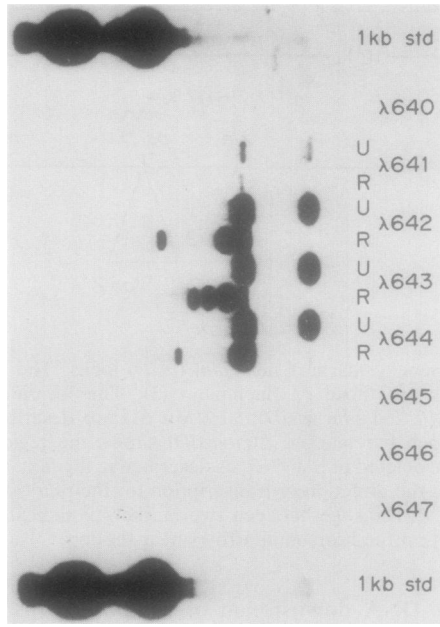

FIG. 2. DNA hybridization of *phn*(EcoB) plasmid with λ *E. coli* K-12 clones. DNAs were separated on a 0.7% agarose gel and tested for hybridization with ^{32}P-labeled pBW120 DNA, as described in Materials and Methods. The top and bottom lanes contain the 1-kb ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), which shows homology with the vector. The central lanes have uncut and *Eco*RI-cut DNAs for λ640, λ641, λ642, λ643, λ644, λ645, λ646, and λ647 (18). U and R denote lanes for uncut and *Eco*RI-cut DNAs that show homology to the probe, respectively.

which was used as a DNA source for the *phn*(EcoB) sequences in the construction of pBW120, as described above.

Structural studies of the *phn* region. An examination was made for gross structural changes in the DNA that could account for the cryptic *phn*(EcoK) DNA, its activating mutations, and the naturally Phn$^+$ *phn*(EcoB) locus. To do this, we digested chromosomal DNAs with several restriction enzymes and compared them by blot hybridization. In brief, DNAs from the *phn*(EcoK) strain BW3912, the *phn*(EcoB) strain NC3, three independent Phn$^+$ *phn*(EcoK) mutants BW9751, BW9752, and BW9753, and the Δ*phn* (*psiD*)*33-30* mutant BW9766 were singly digested with nine enzymes. The same DNAs were also doubly digested with *Bgl*II and *Eco*RV, *Bgl*II and *Pvu*II, *Eco*RI and *Eco*RV, and *Eco*RV and *Hin*dIII. All blots were probed with radiolabeled pBW120 DNA, which contains 15.6-kb *phn*(EcoB) sequences. Chromosomal DNAs for various *phn*(ECOR) alleles were similarly analyzed with a few restriction enzymes.

No restriction site polymorphisms were detected between the *phn*(EcoK) and *phn*(EcoB) DNAs. Importantly, essentially all fragments predicted from the *phn* DNA sequence (Chen et al., in press) were accountable, by size, in blot hybridizations with *phn*(EcoB) DNAs. Only a couple of fragments that were smaller than 0.4 kb were not observed. Interestingly, the blot hybridizations revealed one notable difference between *phn*(EcoK) and *phn*(EcoB) DNAs. A 1.5-kb *Bgl*II-*Eco*RV fragment for *phn*(EcoB) DNA was replaced by a 1.6-kb fragment for the *phn*(EcoK) DNAs, which corresponds in size to DNA flanked by the *Bgl*II$_1$ and *Eco*RV$_1$ sites in Fig. 3. A similar 0.1-kb increase was seen for the corresponding 3.4-kb *Bgl*I$_1$-*Bgl*I$_2$ fragment (in *Bgl*I-digested samples) and the 2.5-kb *Bgl*II$_1$-*Bgl*II$_2$ fragment (in

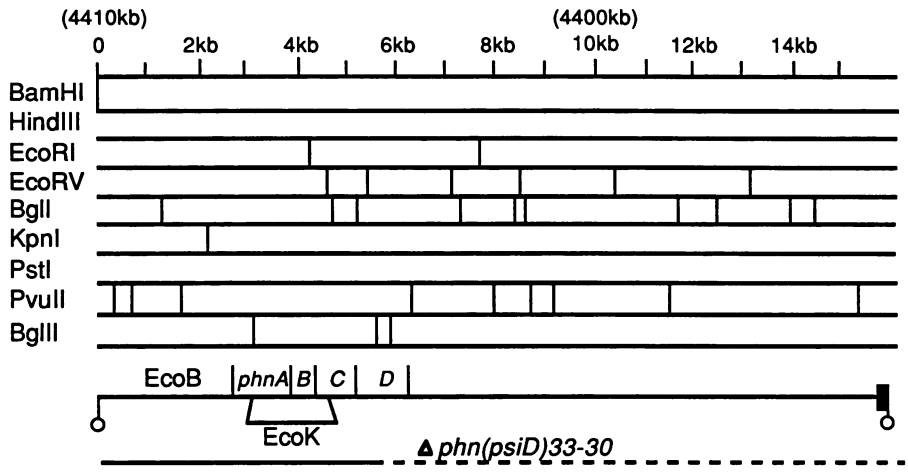

FIG. 3. Structures for the *phn*(EcoB), *phn*(EcoK) and Δ*phn* (*psiD*)*33-30* DNAs as verified by blot hybridization. The restriction map is shown for the 15.6-kb *phn*(EcoB) DNA, as determined by direct DNA sequencing (Chen et al., in press). The numbers in parentheses correspond to kilobase coordinates on the *E. coli* K-12 restriction map (18), near 93 min. The numbered restriction sites in the text refer to their relative positions from left to right, which are marked as vertical lines. The lower line shows the arrangement for the proposed *phnA*, *phnB*, *phnC*, and *phnD* genes. Fourteen additional distal ORFs were identified in the same rightward orientation and named alphabetically as genes *phnE* to *phnQ* (Chen et al., in press). The circles show the *Bam*HI sites that were used to subclone the 15.6-kb *phn*(EcoB) DNA. The solid rectangle on the right denotes Mu DNA from the vector pEG5005, which was used in the initial cloning (see text). EcoK marks the 1.5-kb region that differs for the *phn*(EcoB) and *phn*(EcoK) DNAs. The bottom line shows the extent of the Δ*phn* (*psiD*)*33-30* deletion, where the dashed line marks the deleted region.

both singly *Bgl*II-digested and doubly *Bgl*II- and *Pvu*II-digested samples). We conclude that there exists an additional 0.1 kb in the *phn*(EcoK) sequences within the 3.1- to 4.6-kb interval, as depicted in Fig. 3. No DNA change was seen for the Phn$^+$ *phn*(EcoK) mutants, since each appeared identical to *phn*(EcoK) DNA.

ECORI, which, like *E. coli* K-12, is Phn cryptic, had a pattern similar to *phn*(EcoK), whereas ECOR2, ECOR3, ECOR4, ECOR7, ECOR13, ECOR17, and ECOR20, which show different Phn phenotypes, had unique patterns. Even though both ECOR2 and ECOR17 are naturally Phn$^-$ and could be deleted for *phn* sequences, both had several DNA restriction fragments that hybridized with pBW120 as probe. However, when the 93-min region from ECOR17 was crossed into an *E. coli* K-12 Δ(*phn mel*) mutant with P1, the Mel$^+$ transductants showed very little DNA homology. Apparently, ECOR17 has a deletion of the *phn* sequences near 93-min and other homologous sequences that lie elsewhere. Some other ECOR DNAs also had extra DNA fragments that hybridized with the probe (data not shown).

Our hybridization analysis of DNA from the Δ*phn* mutant BW9766 revealed that the Δ*phn* (*psiD*)*33-30* deletion removes about 20 kb, which appears to be internal to the 30.5-kb chromosomal *Bam*HI fragment near 93 min (18). Although it removes the right two-thirds of the DNA in Fig. 3, the left third appears to be intact. Our actual data are summarized below.

BW9766 DNA showed substantially reduced homology for large (>20-kb) DNA fragments generated with *Bam*HI, *Hin*dIII, *Kpn*I, and *Pst*I. The large *Bam*HI fragment was replaced with a novel 9-kb homologous fragment. The *Pst*I fragment was also noticeably smaller, whereas no reduction in size was noticed for either the *Hin*dIII or *Kpn*I fragments. These data are entirely consistent with a 20-kb deletion, for the remaining *Hin*dIII and *Kpn*I fragments are expected to be large according to the *E. coli* map (18). Other digests allowed us to estimate the left endpoint for the Δ*phn* (*psiD*)*33-30* deletion.

DNA restriction fragments corresponding to the rightmost two *Eco*RI, six *Eco*RV, eight *Bgl*I, seven *Pvu*II, and three *Bgl*II fragments were absent in BW9766 DNAs. The 3.5-kb *Eco*RI$_1$-*Eco*RI$_2$ fragment was replaced with a 4.0-kb fragment that showed reduced homology. Therefore, the deletion endpoint is to the left of the *Eco*RI$_2$ site. New fragments were seen that could account for the loss of the corresponding *Pvu*II$_3$-*Pvu*II$_4$ and *Bgl*II$_1$-*Bgl*II$_2$ fragments, for appropriately singly or doubly digested DNAs, whereas the *Bgl*I$_2$-*Bgl*I$_3$ fragment appeared to be unchanged. These data imply that the deletion ends within the 0.4 kb between the *Bgl*I$_3$ and *Bgl*II$_2$ sites, which would place the left endpoint within the *phnD* ORF (Fig. 3). There was one anomaly in that we failed to detect a new *Eco*RV fragment in place of the missing *Eco*RV$_2$-*Eco*RV$_3$ fragment, which is unexplained. Our interpretation for a left endpoint for the Δ*phn* (*psiD*)*33-30* deletion near the *Bgl*I$_3$ site is consistent with our precisely defining the *phn-33* (*psiD*)::*lacZ*(Mu dl) insertion site in (coincidentally) the *phnD* sequence (W. W. Metcalf, P. M. Steed, and B. L. Wanner, submitted for publication). Accordingly, the Mu dl excision event that formed the Δ*phn* (*psiD*)*33-30* deletion may have removed sequences only to the right of Mu dl, leaving the *phn*::Mu' junction intact.

DISCUSSION

Cryptic genes are silent DNA sequences that can be activated by mutation and for which examples exist for both carbon source catabolism and biosynthesis in bacteria (13). The genes for β-glucoside (Bgl [32]), cellobiose (Cel [31]), citrate (Cit [13]), and threonine (threonine dehydrogenase, Tdh [7]) utilization are normally cryptic in *E. coli* K-12, and their activated forms allow for growth on the respective carbon source. The best-understood mechanism for mutational activation is for the Bgl system. Bgl$^+$ mutants are readily selectable; and they usually occur by a change in the upstream nonencoding region, such as an IS1 insertion, which may act as a transcriptional enhancer (29). Two

cryptic biosynthetic genes code for alternative acetohydroxy acid synthases (AHSs) in branched-chain amino acid biosynthesis: the *ilvG* and *ilvJ* genes in *E. coli* K-12 (34). The wild-type *ilvG*(EcoK) structural gene is silent because it has an early frameshift mutation that abolishes the *ilvG* product AHSII and also reduces somewhat the expression of the distal genes in the *ilvGMEDA* operon (21, 22). The cryptic *ilvG*(EcoK) gene is activatable by frameshift mutations in the *ilvO* region of the *ilvG* gene which restore the proper reading frame; activated IlvG$^+$ mutants are selectable on the basis of the valine insensitivity of AHSII (34). A different class of valine-resistant mutants apparently arise due to activation of the cryptic *ilvJ* gene, which encodes AHSIV (30). The mechanism for *ilvJ* gene activation is unclear.

The *phn* locus defines a new class of cryptic genes, for P assimilation. Like the *bgl*(EcoK) and *ilvG*(EcoK) genes, decryptifying mutations generally lie near the cryptic gene(s) itself. Since both the cryptic *phn*(EcoK) and functional *phn*(EcoB) loci show PhoB-dependent expression, it would seem unlikely that the activating mutations affect the inducibility of the *phn* (*psiD*) operon. It would seem more likely that the structural gene itself is inactive, as is true for the cryptic *ilvG*(EcoK) gene. In this case, decryptifying mutations could act either by restoring the function of the *phn* structural gene or by abolishing polarity on a downstream gene in the *phn* operon.

The *phn*(EcoK) and *phn*(EcoB) genes differ in the 1.5-kb *Bgl*II$_2$-*Eco*RV$_1$ fragment which overlaps sequences for the proposed *phnA*, *phnB*, and *phnC* ORFs (Fig. 3). Further studies are necessary to prove whether this difference in the DNA is responsible for the cryptic *phn*(EcoK) or Phn$^+$ *phn*(EcoB) phenotype. No gross change was detected in three independent activated Phn$^+$ *phn*(EcoK) mutants. In any case, the cryptic *phn*(EcoK) character is common among the ECORs that are closely related to K-12, whereas it is infrequent in other ECORs. Since most *E. coli* are naturally Phn$^+$, the ability to use a Pn as the sole P source is apparently important to *E. coli* in its natural environment.

The *E. coli* K-12 and B strains probably diverged some time ago, because they now exhibit different Phn phenotypes due to a genetic and structural difference in the *phn* locus. We have now shown that allelic differences in the *phn* locus are responsible for differences in the Phn phenotype for some of the ECORs. Also, we noticed differences in the Mel character, which lies nearby. Whereas the *melBA*(EcoK) allele is somewhat Ts$^-$, *E. coli* K-12 transductants with the *melBA*(EcoB) region are not (Wanner and Boline, unpublished data). It will be of interest to define, at the DNA level, the basis for such natural variations near 93 min.

Cryptic genes are thought to play an evolutionary role in nature, especially when a functional gene may have a selective disadvantage (13). Along this line, it is interesting that C-P compounds may have played an important role in the origin of life (47), in which case a gene(s) for Pn utilization could be a vestigial gene(s) from the past. It is noteworthy that the *phn* locus is the first example in which all three possible evolutionary classes of a nonessential gene were identified within the ECOR collection. One class has a cryptic *phn* gene, a second class has a functional Phn$^+$ gene(s), and a third has a nonfunctional, perhaps a deleted, *phn* gene(s).

Even though different biochemical mechanisms may exist for breaking the C-P bond, there is apparently a sole degradative pathway for the metabolism of substituted and unsubstituted phosphonates in *E. coli*. First, nearly all bacteria showed the same Phn phenotype whether tested for utilization of the substituted Pn AEPn or the unsubstituted alkylphosphonates MPn and EPn. Second, all Phn$^+$ mutants of *E. coli* K-12 grew on both Pn types, regardless of which was used in the primary selection. Third, Phn$^-$ mutants of both *E. coli* K-12 and B simultaneously lost the ability to grow on either Pn type. *S. typhimurium* and one ECOR differ in their ability to metabolize various phosphonates. This could reflect a difference in Pn transport or C-P lyase specificity or even the mechanism of C-P bond breakage altogether. It would seem likely that the *phn* locus, which appears to be complex, may encode several inducible genes for P assimilation, including a Pn transport system, C-P metabolizing activity, and possibly others, all of which could normally be cryptic in *E. coli* K-12 but functional in other *E. coli*.

Both the mutationally activated *phn*(EcoK) and the naturally functional *phn*(EcoB) alleles show a phoB-dependent Phn$^+$ phenotype. Since *phn* (*psiD*)::*lacZ* expression requires PhoB, the inability of *phoB* mutants to metabolize a Pn is almost certainly due to decreased *phn* expression. However, *phoB* mutants are pleiotropic and simultaneously abolish the expression of several *phoB*-dependent PHO regulon promoters, including ones for the *phoA* gene, the *pstSCAB-phoU* operon, and the *ugpBAEC*(*psiB/C*) operons (39). Individual mutations in these genes do not affect the Phn phenotype, however (data not shown). Only *phn* mutants are specifically defective in Pn utilization. We conclude that the *phn* locus probably encodes a C-P lyase that cleaves C-P bonds. The *phn* DNA sequence data (Chen et al., in press) suggest that it may also encode a system for Pn transport.

Although Bap can readily hydrolyze various oxygen- or sulfur-linked P compounds to yield P_i, Bap shows no activity toward the C-P (or the N-P) bond (28). Thus, an early report showing that an *E. coli* B strain with a *phoA* mutation had lost its ability to metabolize phosphonates (14) was apparently incorrect. Our studies would indicate that the earlier study (14) may have unknowingly used a *phoB* mutant instead, since it would have displayed both Bap$^-$ and Phn$^-$ phenotypes, as reported.

The *phn* operon lies near 93 min on the genetic map and appears to be transcribed in a counterclockwise orientation. Very few known genes lie in this region of the chromosome. The nearest one for which linkage data exist is the *mel* operon, which lies more than 30 kb away. A complex structure for the *phn* locus is inferred from both our molecular cloning and genetic data. Of eight independent Tn*5*- or Tn*10*-induced Phn$^-$ mutations, all lie near 93 min. It is therefore unlikely that any (nonessential) *phn* structural gene lies elsewhere on the chromosome. Yet our *phn*::Tn*5* and Tn*10* mutations show different amounts of genetic linkage with our *phn* (*psiD*)::*lacZ*(Mu d1) mutations, which supports our results from cloning experiments and implies that the *phn* operon is quite large. Individual Phn$^-$ mutations may span a region of about 10 kb on the chromosome (Metcalf and Wanner, unpublished data). Our characterization of the *E. coli phn* locus provides the groundwork for future studies on the molecular genetics and biochemistry of Pn metabolism and its regulation in bacteria.

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