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Molecular Cloning, Mapping, and Regulation of Pho Regulon Genes for Phosphonate Breakdown by the Phosphonatase Pathway of *Salmonella typhimurium* LT2

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Two pathways exist for cleavage of the carbon-phosphorus (C-P) bond of phosphonates, the C-P lyase and the phosphonatase pathways. It was previously demonstrated that *Escherichia coli* carries genes (named *phn*) only for the C-P lyase pathway and that *Enterobacter aerogenes* carries genes for both pathways (K.-S. Lee, W. W. Metcalf, and B. L. Wanner, J. Bacteriol. 174:2501–2510, 1992). In contrast, here it is shown that *Salmonella typhimurium* LT2 carries genes only for the phosphonatase pathway. Genes for the *S. typhimurium* phosphonatase pathway were cloned by complementation of *E. coli* Δ*phn* mutants. Genes for these pathways were proven not to be homologous and to lie in different chromosomal regions. The *S. typhimurium phn* locus lies near 10 min; the *E. coli phn* locus lies near 93 min. The *S. typhimurium phn* gene cluster is about 7.2 kb in length and, on the basis of gene fusion analysis, appears to consist of two (or more) genes or operons that are divergently transcribed. Like that of the *E. coli phn* locus, the expression of the *S. typhimurium phn* locus is activated under conditions of P_i limitation and is subject to Pho regulon control. This was shown both by complementation of the appropriate *E. coli* mutants and by the construction of *S. typhimurium* mutants with lesions in the *phoB* and *pst* loci, which are required for activation and inhibition of Pho regulon gene expression, respectively. Complementation studies indicate that the *S. typhimurium phn* locus probably includes genes both for phosphonate transport and for catalysis of C-P bond cleavage.

Phosphonates (Pn) are a large class of molecules containing a direct carbon-phosphorus (C-P) bond in place of the more familiar carbon-oxygen-phosphorus bond of phosphoesters. Although Pn do not exist in all organisms, they do exist in many different ones. Pn have been found in organisms as diverse as the intracellular procaryote *Bdellovibrio* species, the filamentous bacteria streptomycetes, the protozoans *Tetrahymena* and *Trypanosoma* spp., mollusks, insects, and others. In these organisms, Pn have been isolated as constituents of glycolipids, glycoproteins, polysaccharides, or phosphonolipids. In *Bacteroides fragilis*, Pn were identified as components of capsular polysaccharide (6); in streptomycetes, Pn are synthesized as antibiotics such as fosfomycin (25); in *Tetrahymena* spp., Pn exist as phosphonolipids (19); in *Trypanosoma cruzi*, Pn are components of lipopeptidophosphoglycan (the major cell surface glycoconjugate [10]); in a sea anemone, Pn are the major P compounds (38); and in a locus, a Pn is the principal P compound of hemolymph (20). Yet, in spite of their widespread natural occurrence, the biological role of natural Pn is poorly understood (17). In phosphonolipids, 2-aminoethylphosphonate (AEPn) exists in place of its analog ethanolamine phosphate.

No doubt because of the abundance of PN in nature, bacteria have acquired pathways for Pn breakdown. Bacteria capable of breaking the C-P bond may use Pn as a sole P source, for which two pathways exist, the C-P lyase and the phosphonatase pathways (Fig. 1) (47). These pathways differ in regard to both their substrate specificity and the catalytic mechanism of C-P bond fission. The C-P lyase pathway has a broad substrate specificity. It leads to cleavage of substituted Pn (such as AEPn) as well as unsubstituted Pn (such as methylphosphonate) by a mechanism involving redox or radical chemistry (Fig. 1A). As a result of the absence of a biochemical assay for the C-P lyase pathway, its mechanism of C-P bond cleavage is poorly understood. The phosphonatase pathway, by comparison, has a narrow substrate specificity. It acts primarily on naturally occurring AEPn. In a two-step process, the phosphonatase pathway leads to cleavage of the C-P bond by a hydrolysis reaction requiring an adjacent carbonyl group (Fig. 1B). AEPn is converted to phosphonoacetaldehyde by an AEPn-specific transaminase, and phosphonoacetaldehyde is subsequently converted to acetaldehyde and P_i by phosphonoacetaldehyde hydrolase (trivial name, phosphonatase). Enzymes carrying out both of these steps have been characterized for *Bacillus cereus* (26) and *Pseudomonas aeruginosa* (12, 23). Uptake systems specific for AEPn have also been demonstrated for these bacteria (22, 39), consistent with breakdown occurring in the cytoplasm.

Our interest in Pn breakdown resulted from the discovery that the synthesis of C-P lyase activity is under Pho regulon control in *Escherichia coli* (46). Subsequent studies showed that genes for the C-P lyase pathway are in an operon of 14 genes named (in alphabetical order) *phnC* to *phnP* (9, 36, 51). Three of these genes (*phnC*, *phnD*, and *phnE*) probably encode a binding protein-dependent Pn transporter; two (*phnF* and *phnO*) appear to encode gene regulatory proteins, and nine others (*phnG* to *phnN* and *phnP*) probably encode proteins required for C-P bond cleavage, which may exist as a membrane-associated C-P lyase enzyme complex (33, 34; reviewed in references 47, 50, and 54). Nevertheless, in spite of the wealth of information on the molecular biology and genetics of genes for the C-P lyase pathway, it has not been possible to detect C-P lyase activity in a cell-free system.

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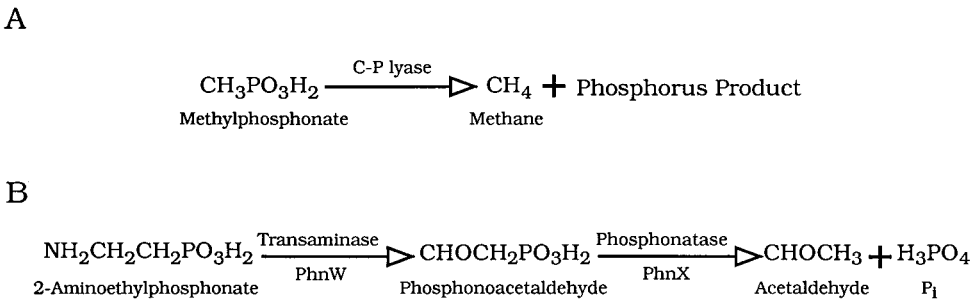

FIG. 1. Pathways of carbon-phosphorus bond cleavage. (A) The C-P lyase pathway acts on alkyl- or arylphosphonates as well as on Pn having a substitution on the carbon (such as AEPn). The carbon product resulting from breakdown of an alkylphosphonate by the C-P lyase pathway is the corresponding alkane, while the phosphorus product is unknown. At least seven genes (named *phnG* to *phnM*) appear to be required for the C-P lyase activity; in addition, *phnN* and *phnP* may encode C-P lyase accessory proteins. (B) The primary substrate of the phosphonatase pathway is AEPn, which is broken down in a two-step reaction, releasing acetaldehyde and P_i as coproducts (47). The genes encoding the transaminase and phosphonatase have been named PhnW and PhnX, respectively (31).

With an eventual goal of developing an in vitro system for detection of C-P lyase activity, we cloned new genes for Pn degradation from *Enterobacter aerogenes* (24). We had expected *Enterobacter aerogenes* to provide a more accessible C-P lyase activity, although this was later proven to be wrong (47). *Enterobacter aerogenes* is also capable of using a broader range of Pn substrates as a sole P source than *E. coli* is. We unexpectedly found *Enterobacter aerogenes* to carry genes for two separate Pn degradation pathways. It carries genes homologous to those for the C-P lyase pathway of *E. coli*; however, the genes for Pn transport and C-P lyase activity appear to be unlinked in *Enterobacter aerogenes*. In addition, *Enterobacter aerogenes* carries genes for the phosphonatase pathway. Genes for the C-P lyase and phosphonatase pathways of *Enterobacter aerogenes* are unlinked to each other and have no homology detectable by DNA hybridization. Although genes for Pn transport are linked to genes for the phosphonatase pathway, the transporter associated with the phosphonatase pathway appears to be specific for AEPn, the substrate of that pathway.

Furthermore, like genes of the *E. coli* C-P lyase pathway, genes for both Pn degradation pathways of *Enterobacter aerogenes* appear to be under Pho regulon control. Our finding genes under Pho regulon control for Pn degradation by two different pathways in a single organism highlights the importance of Pn as a nutrient in nature. It was also surprising to find the phosphonatase pathway in a member of the family *Enterobacteriaceae*. Until recently, the view that, at least among commonly studied bacteria, the C-P lyase pathway was prevalent only among gram-negative bacteria and the phosphonatase pathway was prevalent only among gram-positive bacteria had been widely held. The finding of the phosphonatase pathway in *P. aeruginosa* (11) was an unremarkable exception because *Pseudomonas* species often exhibit diverse metabolic capabilities (18).

We previously reported that *Salmonella typhimurium* LT2 differs from many closely related bacteria (including *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Proteus*, *Serratia*, and *Shigella* species) in that *S. typhimurium* is capable of using AEPn and no other Pn as a sole P source (51). Although such differences may have been attributable solely to transport specificity, the ability to break down only AEPn is also characteristic of the phosphonatase pathway. Therefore, we cloned genes for AEPn utilization from *S. typhimurium*. We found them to be homologous to those for the phosphonatase pathway from *Enterobacter aerogenes*. We also showed that as expected of genes with a primary role in P assimilation, these genes are under Pho regulon control in *S. typhimurium*. These studies of the *S. typhimurium phn* locus are reported here.

MATERIALS AND METHODS

Media, chemicals, and other materials. Most of the materials used in this study have been described elsewhere (48, 51). Media buffered with 3-[*N*-morpholino]propanesulfonic acid (MOPS) and containing 2 mM P_i, 0.1 mM P_i, or 0.5 mM AEPn were used to study P_i regulation or for growth with AEPn as a sole P source. In most cases, MOPS agar containing AEPn was prepared with Bacto Agar (Difco Laboratories, Detroit, Mich.). The small amounts of contaminating P_i provided faint background growth, above which Pn^+ transductants or transformants were easily seen. Most antibiotics and chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were used at the following concentrations: ampicillin at 25 or 100 μg/ml for single-copy or multicopy resistance genes, chloramphenicol at 25 μg/ml, kanamycin at 50 μg/ml, spectinomycin and streptomycin at 35 μg/ml each, and tetracycline at 15 μg/ml. Streptomycin was used at 100 μg/ml when selecting for resistance due to a chromosomal *rpsL* allele.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl-*p*-toluidine (XP) are the blue dyes for detection of β-galactosidase and nonspecific phosphatases, respectively. These were obtained from Bachem (Torrance, Calif.) and were added at 40 μg/ml. EBU agar contains 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 2.5 g of glucose, 2.5 g of K_2HPO_4, 1.25 ml of a 1% solution of Evans blue, 2.5 ml of a 1% solution of uranine (sodium fluorescein), and 15 g of agar per liter and was used for routine purification of all transductants.

Bacteria, phage, and plasmids. The bacteria used are listed in Table 1 or described below. Those with BW and TBW prefixes are derivatives of *E. coli* K-12 and *S. typhimurium*, respectively. A set of *S. typhimurium* strains carrying locked-in Mu*d*-P22 prophages (7) was obtained from N. Benson. A membrane impregnated with DNAs in an ordered array from induced lysates of a subset of these strains was provided by K. K. Wong and M. McClelland. A set of mitomycin-induced lysates of them and P22 tail protein was provided by M. Laves and S. Maloy. Several additional strains that were used primarily in preliminary mapping experiments were provided by K. E. Sanderson. *S. typhimurium* CDC 6516-60 (ATCC 14028) was obtained from J. Mekalanos.

pBC10 has the 4.7-kbp *Eco*RI-to-*Pst*I fragment containing the *E. coli* K-12 *phoBR* operon from pBC6 (52) cloned into pBR322 (8). pBW120 carries the entire *phnC*-to-*phnP* gene cluster from *E. coli* B cloned into pUC18 (51). pEG5166-KL38 and pEG5166-KL124 carry genes from *Enterobacter aerogenes* for the phosphonatase and C-P lyase pathways, respectively (27). pWM4, pWM5, and pWM6 contain cassettes with a promoterless *uidA* and different antibiotic resistance markers (35). The sources of pEG5294 (32) and pSKS114 (55) have been reported. λRZ5*phoA-lacZ*$_{DK110}$ has a transcriptional fusion of *lacZ* to the *E. coli phoA* promoter (2). P1 and P22 refer to P1*kc* and P22*ht105 int-103*, respectively. A P22 lysate prepared on a pool of random Tn*10* mutants of *S. typhimurium* LT2 was obtained from P. Youderian.

pWM7 (30) has the γ replication origin of the R6K plasmid (*ori*R_{R6K_γ}), and so it is capable of replicating only in strains (such as BW16945, BW17272, BW19615, or BW19094 [Table 1]) in which the II protein (the *pir* gene product) is provided in *trans*. This plasmid also carries the transfer origin (*oriT*) of the RP4 plasmid, thus allowing it to be transferred by conjugation from a host (such as BW17272) providing both II and *trans*-acting transfer functions. In addition, pWM7 carries *tetAR* for tetracycline resistance (Tet^r) from Tn*10*, which provided a means to counterselect recombinants carrying derivatives of pWM7 integrated in the chromosome. pWM41 and pWM75 have the f1*ori* and *lacZ*α regions of pBluescript II SK(+) or pBluescript II KS(+) (obtained from Stratagene, La

TABLE 1. Bacterial strains

Strain[a]	Genotype	Pedigree[b]	Source
E. coli K-12 derivatives			
BW5104	Mu-1 Δ*lac-169 creC510 hsdR514*	BD792 via BW4714	32
BW10843	Δ*lac-169* Δ(*proC aroLM phoB519*)*4*::Tn*5-132 creC510 thi*	XPh4 via BW10820	This study
BW14329	Mu-1 Δ*lac-169* Δ(*mel proP phnCDEFGHIJKLMNOP*)*2*::Tn*5seq1/132* (*tet*) Δ*phoA532 creC510 hsdR514*	BD792 via BW14295	24
BW14648	Mu-1 Δ*lac-169* Δ(*phoBR*)*525 creC510 hsdR514*	BD792 via BW14277	This study
BW16787	Mu-1 Δ*lac-169* Δ(*phnHIJKLMNOP*)*4/2*::Tn*5-132* Δ*phoA532 creC510 hsdR514*	BD792 via BW14329	24
BW16945	λ*pir* Δ*lac-169* Δ(*phnHIJKLMNOP*)*4/2*::Tn*5-132 creC510 hsdR514 rpsL*	BD792 via BW15818	This study
BW17272	λ*pir* RP4-2 *tet*::Mu-1*kan*::Tn*7* integrant *recA*::*cat-aadA creC510 hsdR17 endA1 zbf-5*::Tn*10 thi*	S17-1 via BW17268	34
BW19094	DE3(*lac*)X74 Δ*phoA532* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 uidA*(Δ*Mlu*I)::*pir*$^+$	BD792 via BW16878	30
BW19615	DE3(*lac*)X74 Δ*phoA532* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 uidA*(Δ*Mlu*I)::*pir*$^+$ *rpsL*	BD792 via BW19094	30
BW19712	DE3(*lac*)X74 *phn*(EcoB) Δ(*phoBR*)*525 uidA*(Δ*Mlu*I)::*pir*$^+$ *rpsL lamB*	BD792 via BW19654	λ*vir*^{r} Mal$^+$
BW19715	DE3(*lac*)X74 Δ*phoA532* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 uidA*(Δ*Mlu*I)::*pir*$^+$ *rpsL malT*	BD792 via BW19615	30
TE1335	F′128[*pro*(*BA*)$^+$ *lac*(*ZYA*)$^+$]::P22*ht105 int-201 sieA44*/DE3(*lac*)X74 *rpsL trp*	K-12	T. Elliott
S. typhimurium derivatives			
AK3029	F′112 *metB*$^+$ *malB*$^+$/LT2 *zai-3029*::Tn*10-11*(*tet*) *trpC2 metA22 metE551 his-6165 ilv-452 galE496* Fels2$^-$ *rpsL120 hsdL6 hsdSA29*	LT2 via TS736	C. Miller via K. E. Sanderson
JF511	LT2 Δ*nadA100 psiA9*::*lacZ*(Mu d1)	LT2	J. Foster
JF512 (SGSC1331)	LT2 Δ*nadA100 psiB12*::*lacZ*(Mu d1)	LT2	K. E. Sanderson
JF515	LT2 Δ*nadA100 phn-128* (*psiC17*)::*lacZ* (Mu d1)	LT2	J. Foster
JF663 (SGSC1335)	LT2 *leu-515*(Am) *su-19 nadA*::Tn*10 psiD19*::*lacZ* (Mu d1-8)	LT2	K. E. Sanderson
MS1868	LT2 *leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$	LT2	N. Benson
MS509	LT2 *phoP53*::Tn*10-11*(*tet*)	LT2	S. Maloy
PY13666	LT2 *leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$ *hisD9953*::Mu d1-1734)	LT2 via MS1868	P. Youderian
SL1657	LT2 *trpC2 metA22 ilvC2 metE551 ilv-452 xyl-404 galE496 hsdL6 hsdSA29 rpsL120* H1(*b*) H2(*enx*) *fla-66 nml* Fels2$^-$	LT2	K. E. Sanderson
TBW13740	LT2 λRZ*5phoA-lacZ*$_{DK110}$ *trpC2 metA22 ilvC2 metE551 ilv-452 xyl-404 galE496 hsdL6 hsdSA29 rpsL120* H1(*b*) H2(*enx*) *fla-66 nml* Fels2$^-$	LT2 via SL1657	This study[c]
TBW13842	LT2 λRZ*5phoA-lacZ*$_{DK110}$ *pst-4*::Tn*10 trpC2 metA22 ilvC2 metE551 ilv-542 xyl-404 galE496 hsdL6 hsdSA29 rpsL120* H1(*b*) H2(*enx*) *fla-66 nml* Fels2$^-$	LT2 via TBW13741	Tet^r with P22 on Tn*10* pool[c]
TBW19327	LT2 *leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$ *recD541*::Tn*10d*CM	LT2 via MS1868	Cm^r with P22 on TT16812
TBW19329	LT2 *leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$ *recD543*::Tn*10d*Tet	LT2 via MS1868	Tet^r with P22 on TT16814
TBW19632	LT2 *phn-108*::*uidA2-aphA leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$ *recD541*::Tn*10d*CM	LT2 via TBW19327	Kan^r with *Bam*HI-cut pWM65
TBW19800	LT2 Δ*phoB1*::*cat leuA414*(Am) *hsdSB* (r$^-$ m$^+$) Fels2$^-$ *recD543*::Tn*10d*Tet	LT2 via TBW19329	Cm^r with *Bam*HI-cut pWJ6
TBW19801	LT2 *phoB2*::*cat leuA414*(Am) *hsdSB* (r$^-$ m$^+$) Fels2$^-$ *recD543*::Tn*10d*Tet	LT2 via TBW19329	Cm^r with *Bam*HI-cut pWJ7
TBW19812	LT2 Δ*phoB1*::*cat leuA414*(Am) *hsdSB* (r$^-$ m$^+$) Fels2$^-$	LT2 via MS1868	Cm^r with P22 on TBW19800
TBW19912	LT2 *pst-4*::Tn*10 leuA414*(Am) *hsdSB*(r$^-$ m$^+$) Fels2$^-$	LT2 via MS1868	Tet^r with P22 on TBW13842
TBW19913	LT2 *leu-515*(Am) *su-19 nadA*::Tn*10 psiD19*::*lacZ* (Mu d1-1734)	LT2 via JF663	Kan^r with P22 on PY13666[c]
TBW19914	LT2 Δ*nadA100 psiB12*::*lacZ* (Mu d1)	LT2 via JF512	Kan^r with P22 on PY13666[c]
TBW19915	LT2 Δ*nadA100 phn-128* (*psiC17*)::*lacZ* (Mu d1-1734)	LT2 via JF515	Kan^r with P22 on PY13666[c]
TBW19953	LT2 *hsdSB*(r$^-$ m$^+$) Fels2$^-$	LT2 via MS1868	Leu$^+$ with P22 on AK3029
TBW20103	LT2 Δ*nadA100 psiA9*::*lacZ* (Mu d1-1734)	LT2 via JF511	Kan^r with P22 on PY13666[c]
TL156	LT2 Fels2$^-$ *galE496 metA22 metE55 rpsL120 xyl404* H1(*b*) H2(*enx*) *nml ilv hsdL6 hsdSA29* [Muc*62*(Ts$^-$)*h7629*]		L. Csonka
TT13206	LT7 *phoN51*::Tn*10-11*(*tet*)	LT7	N. Zhu and J. Roth
TT16812	LT2 *recD541*::Tn*10d*CM	LT2 via TH2029	J. Roth via K. T. Hughes
TT16814	LT2 *recD543*::Tn*10d*Tet	LT2 via TH2029	J. Roth via K. T. Hughes

[a] All *E. coli* strains are derivatives of *E. coli* K-12, except that the *phn*(EcoB) locus is from *E. coli* B (51).
[b] Pedigree gives the parental strain from another laboratory and its most immediate ancestor in this or another laboratory.
[c] See Materials and Methods.

Jolla, Calif.) cloned into the *Bam*HI site of pWM7 such that the site was destroyed (29). pWM75 has an *Nsi*I deletion of *tetAR*.

Molecular genetics. Transductions with P1 (48) or P22 (28) were carried out as described previously. Transductants were purified routinely at least once on EBU agar prior to testing phenotypes. Pn phenotypes were always tested by growing colonies on glucose-MOPS agar containing 2 mM P_i beforehand and by comparing them side by side with appropriate Pn$^+$ and Pn$^-$ control strains on the same plate. TBW13740 was isolated as an ampicillin-resistant (Amp^r) and streptomycin-resistant (Str^r) exconjugant displaying a phosphate-starvation-inducible (Psi) Lac$^+$ phenotype after mating of SL1657 with a λRZ*5phoA-lacZ*$_{DK110}$ lysogen of the HfrH derivative CA7027 (1). TBW13740 probably has the phage integrated in *att*λ of *S. typhimurium*. Upon transduction to Gal$^+$ or

tetracycline resistance with P22 grown on MS1868 (Table 1) or TT403 (*bio-102*::Tn*10*; from J. Roth via K. E. Sanderson), 14.5% (25 of 172) or 72.8% (302 of 415) of the TBW13840 transductants simultaneously became Lac$^-$ and Amp^s, respectively. TBW13842 is a Lac$^+$ mutant of TBW13740 that was selected as a dark-blue, Tet^r transductant on X-Gal agar containing excess P_i after infection with P22 grown on a pool of Tn*10* mutants. It probably has a *pst* mutation (designated the *pst-4*::Tn*10* allele) because its Lac$^+$ character (on medium containing excess P_i) is linked to the *ilv* locus, as expected by analogy to the location of the *pstSCAB-phoU* operon of *E. coli.*

The *psiA9*, *psiB12*, *phn-128* (*psiC17*), and *psiD19*::*lacZ* (Mu d1) insertions of JF511, JF512, JF515, and JF663 (Table 1) were converted to their respective Mu d1 1734 (also called Mu dJ) insertions with P22 grown on PY13666. Kanamycin-resistant (Kan^r) and His$^+$ transductants were tested for Amp^s ones showing the parental Lac phenotype. These transductants were used for preparation of P22 lysates to cross each of these insertions into MS1868, TBW19812 (MS1868 Δ*phoB1*::*cat*), and TBW19912 (MS1868 *pst-4*::Tn*10*).

Molecular biology. Most molecular biology and recombinant DNA techniques were carried out as described previously (51) or by use of standard procedures (3, 41). Enzymes and kits were obtained from Boehringer Mannheim (Indianapolis, Ind.), Gibco BRL (Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), and Promega (Madison, Wis.) and were used as described in the suppliers' recommendations. Transformation of *S. typhimurium* was carried out by electroporation with a Cell-Porator with a voltage booster (Gibco BRL). Transformation of *E. coli* was carried out by standard $CaCl_2$ treatment or electroporation.

Cloning of *S. typhimurium* genes. TBW14245 is a derivative of the *S. typhimurium* LT2 strain TL156 (Table 1) carrying pEG5294 (15). It was isolated as an Amp^r transductant with a P22 lysate of an *E. coli* K-12 strain, BW5104, carrying pEG5294. This P22 lysate resulted from zygotic induction after mating of TE1335 (13) with a pEG5294 transformant of BW5104 as described elsewhere (13). Mini-Mu plasmid lysates of TBW14245 were prepared by heat induction, as reported previously (51). These were used to infect Mu-1 lysogens of various Hsd$^-$ *E. coli* mutants, after which Kan^r transductants were selected and then replica-plated onto glucose-MOPS agar containing AEPn and kanamycin to find AEPn$^+$ ones. pEG5294-MK4 was isolated from an AEPn$^+$ transductant of the Δ*phn* mutant BW14329, and pEG5294-MK8, pEG5294-MK10, and pEG5294-MK11 were isolated from AEPn$^+$ transductants of the Δ*phn* mutant BW16787. pEG5294-MK4, pEG5294-MK8, and pEG5294-MK10 are also called pKL242, pKL245, and pKL246, respectively.

pKL248, pKL253, and pKL257 carry a 5.5-kbp *Bam*HI fragment from pEG5294-MK8, a 2.9-kbp *Bam*HI fragment from pEG5294-MK10, and a 5.0-kbp *Bam*HI fragment from pEG5294-MK11 cloned into pWM7, respectively. Transductants of the λ*pir* Δ*phn* mutant BW16945 were selected as AEPn$^+$ ones. pWM61 and pWM62 carry a ca. 14.9-kbp fragment generated by partial digestion of pEG5294-MK10 with *Bam*HI cloned into the polylinker of pWM41 in opposite orientations. This 14.9-kbp fragment was subcloned as an *Apa*I-to-*Sac*I fragment from these plasmids into pWM75 to construct pWM67 and pWM68, respectively. This was feasible because these sites flank the *Bam*HI site of the pWM41 polylinker and are absent from the insert (data not shown). AEPn$^+$ transformants of the *pir*$^+$ Δ*phn* mutant BW19094 were selected. pWM64, pWM65, and pWM66 carry the 3.8-kbp *Bam*HI *uidA2-aadA* cassette of pWM5, the *uidA2-aphA* cassette of pWM6, and the *uidA2-cat* cassette of pWM4, respectively, cloned into the *Bgl*II site of pWM60. They were identified as plasmids capable of expressing *uidA* in response to P_i limitation. As expected, each has the *uidA* cassette in the same orientation. Because these insertions resulted in an AEPn$^-$ phenotype, insertions at this *Bgl*II site were given the allele designation *phn-108.*

pEG5294-KL288 and pEG5294-KL292 probably carry the *proC*-to-*phoBR* region of *S. typhimurium*. They were isolated from Kan^r and XP$^+$ (dark-blue) transductants of the Δ*phoBR* mutant BW14648 on glucose-MOPS agar containing 0.1 mM P_i. Because XP is a nonspecific phosphatase substrate, only those XP$^+$ colonies producing alkaline phosphatase activity when tested with *p*-nitrophenylphosphate (as described elsewhere [48]) were considered as candidates for *phoB*$^+$ ones. Transductants presumed to contain *phoB*$^+$ plasmids were tested for ones likely to carry *proC*$^+$ on the plasmid, because *phoB* and *proC* are closely linked in *E. coli*. To do this, 12 presumptive *phoB*$^+$ transductants of BW14648 were made Tet^r with a P1 lysate of the Δ(*proC-phoB*)::Tn*5-132* mutant BW10843 (Table 1). Because all Tet^r transductants of BW14648 carrying pEG5294-KL288 or pEG5294-KL292 were Pro$^+$, *proC*$^+$ is probably carried by these plasmids. BW14648 carrying any of 10 other presumptive *phoB*$^+$ plasmids yielded only Tet^r transductants that were Pro$^-$, indicating that they did not carry *proC*. BW10843 has a *proC*-to-*phoB* deletion that resulted from substitution of the *proC*-to-*phoBR* interval with the tetracycline resistance element Tn*5-132*, as described elsewhere (33).

pWJ1 and pWJ3 were made by cloning 1.8-kbp *Nsi*I and 6.7-kbp *Pst*I fragments from pEG5294-KL288 into *Nsi*I-digested pWM7, respectively. pWJ2 and pWJ4 were made by cloning similar fragments from pEG5294-KL292 into *Nsi*I-digested pWM7. All of these plasmids were isolated from XP$^+$ transformants of the *pir*$^+$ Δ*phoBR* mutant BW19655. Because the *Pst*I fragments in these plasmids contain two *Sal*I sites while the 1.8-kbp *Nsi*I fragments contain a single *Sal*I site, a *Sal*I site may lie in *phoB*. To construct *phoB* mutations, pWJ3 was partially digested with *Sal*I and ligated with the 1.9-kbp *Sal*I *cat* fragment from pSKS114. Three types of plasmids (pWJ5, pWJ6, and pWJ7) unable to complement a *phoB* mutant were found. pWJ6 (Δ*phoB1*::*cat*) has a deletion of a 2.1-kbp *Sal*I fragment, and pWJ7 (*phoB2*::*cat*) has a simple insertion.

Mutagenesis. Transposon mutagenesis was carried out with λ suicide vectors carrying Tn*phoA* (capable of forming *phoA* gene fusions), Tn*phoA′-1* (capable of forming *lacZ* transcriptional fusions), or Tn*phoA′-4* (capable of forming *lacZ* translational fusions) essentially as described elsewhere (48, 55). To do this, BW17272 carrying pKL248, pKL253, pKL257, or pWM67 was infected with one of these phages, Kan^r transductants were selected, and these were replica-mated with BW16945, BW19615, or BW19715. Kan^r and Str^r exconjugants of them were selected by replica-plating the mating mixtures onto agar containing kanamycin and streptomycin. Several well-isolated colonies were purified and tested for AEPn$^-$ ones. Seven independent mutants (designated as carrying the *phn-101* to *phn-107* alleles) were isolated following Tn*phoA′-1* mutagenesis of pKL248, pKL253, or pKL257, and 19 independent mutants (designated as carrying the *phn-109* to *phn-127* alleles) were isolated following Tn*phoA*, Tn*phoA′-1*, or Tn*phoA′-4* mutagenesis of pWM67. The orientation and approximate locations of the insertions were determined by restriction mapping (data not shown).

pWJ3 was similarly subjected to transposon mutagenesis. BW17272 carrying pWJ3 was infected with λ::Tn*phoA′-4*, after which Kan^r transductants were selected and then replica-mated with BW19712. Kan^r and Str^r exconjugants were selected on agar containing XP, and plasmid DNAs were isolated from non-blue ones. One with Tn*phoA′-4* inserted in the *phoB* segment was designated as carrying the *phoB3* allele.

Allele replacement. Two methods of allele replacement were used. One method was dependent upon the use of $oriR_{R6K\gamma}$ plasmids carrying *tetAR* and was carried out essentially as described previously (30). In brief, an *E. coli* strain capable of replication and conjugative transfer of these plasmids (BW17272 [Table 1]) was transformed with mutant plasmids carrying the *phn-101* to *phn-107*::Tn*phoA′-1* alleles, and these transformants were then mated with SL1657 and Kan^r and Str^r exconjugants were selected. Because of the inability of $oriR_{R6K\gamma}$ plasmids to replicate in the recipient (SL1657), exconjugants arise by integration of the plasmid into the chromosome by homologous recombination. Several resultant exconjugants were purified once nonselectively, after which Tet^s recombinants were selected and then tested for kanamycin resistance, AEPn phenotype, and blue color on X-Gal agar (under conditions of P_i limitation).

The other method involved transformation of a *recD* mutant with linear DNA fragments (40). All three *phn-108*::*uidA* insertions were recombined onto the chromosome by selection of antibiotic-resistant transformants of the *recD* mutant TBW19327 with linear DNAs prepared by digestion of pWM64, pWM65, and pWM66 with *Bam*HI. Likewise, the *phn-116*::Tn*phoA′-1* allele was recombined onto the chromosome by selecting Kan^r transformants of TBW19329 with *Apa*I-digested pWM67*phn-116*::Tn*phoA′-1*; the *phoB3*::Tn*phoA′-4* mutation was recombined onto the chromosome by selecting Kan^r transformants of TBW19329 with *Eco*RI-digested pWJ3*phoB3*::Tn*phoA′-4*; the Δ*phoB1*::*cat* and *phoB2*::*cat* alleles were recombined onto the chromosome by selecting chloramphenicol-resistant (Cm^r) transformants of the *recD* mutant TBW19329 with *Bam*HI-digested pWJ6 and pWJ7, respectively.

Cell growth. Broth cultures were inoculated with single colonies freshly grown on 0.2% glucose-MOPS agar containing 2 mM P_i and required supplements. Colonies were inoculated into 0.06% glucose–MOPS medium containing 2 mM P_i (for excess-P_i cultures) or 0.4% glucose–MOPS medium containing 0.1 mM P_i (for limited-P_i cultures) and incubated at 37°C with aeration for about 16 h prior to sampling for enzyme assays. SL1657 derivatives were grown in media supplemented with L-methionine, L-tryptophan, L-isoleucine, and L-valine; MS1868 derivatives were grown with L-leucine; and TBW19953 derivatives were grown without additional supplements.

Enzyme assay. β-Galactosidase activities were usually assayed with cells treated with sodium dodecyl sulfate and chloroform as described elsewhere (48). The results of these assays are expressed as units (nanomoles of product formed per minute) per cell culture optical density at 420 nm. β-Glucuronidase (also known as GUS, encoded by *uidA*) activities were measured with sonic cell extracts. Cells from 10-ml cultures were collected by centrifugation and resuspended in 0.5 ml of assay buffer (50 mM sodium phosphate [pH 7.0], 10 mM 2-mercaptoethanol, 100 μg of chloramphenicol per ml). Cells were broken with a model 450 sonifier (Branson, Danbury, Conn.) with a 4.8-mm tapered microtip at 20 W for 4.5 min with a 30% duty cycle, while sample tubes were held in an ice-water bath. The assays were carried out at 37°C with 1 ml of assay buffer and 25 to 200 μl of extract. The assays were initiated by the addition of 0.1 ml of 0.4% *p*-nitrophenyl-β-D-glucuronide, and they were stopped by the addition of 0.5 ml of 2.5 M 2-amino-2-methylpropanediol and placement on ice. The A_{415} was determined, and units were calculated by use of the molar extinction coefficient E_{415} of 1.62×10^4 for *p*-nitrophenol. The results of assays determined with sonic cell extracts are expressed as units per milligram of protein. Protein was measured by the Lowry method with bovine serum albumin as a standard (41).

RESULTS

Cloning genes for the phosphonatase pathway from *S. typhimurium*. We cloned genes for Pn utilization from *S. typhimurium* by the mini-Mu technique by complementation of *E. coli* Δ*phn* mutants. Because *E. coli* has only the C-P lyase path-

way, *E. coli* mutants with mutations of the *phnCDEFGHJKLM NOP* locus are unable to use any Pn as a P source (51). Two *E. coli* Δ*phn* mutants were used to isolate complementing plasmids. One mutant (BW14329) is deleted of the entire *phnC*-to-*phnP* gene cluster, and so only those plasmids expressing genes for both Pn transport and Pn breakdown are expected to complement this mutant. The other mutant (BW16787) has an intact Pn transporter (encoded by *phnCDE*) and is deleted of the *phnH*-to-*phnP* segment, and so plasmids expressing only genes for Pn breakdown are expected to complement this mutant. $AEPn^+$ transductants were selected following infection with mini-Mu plasmid lysates prepared from the *S. typhimurium* LT2 strain TBW14245. Two transductants of BW14329 and seven transductants of BW16787 that carry plasmids capable of complementing these mutants were found. This was shown by isolating plasmid DNAs from several $AEPn^+$ transductants and using these DNAs to isolate Kan^r transformants of BW14329 and BW16787. Both plasmids capable of complementing BW14329 were also capable of complementing BW16787, as expected. One of these (pKL242) was used in subsequent studies. Only two plasmids selected for complementation of BW16787 were also capable of complementing BW14329. One plasmid capable of complementing only BW16787 was named pKL245, and one capable of complementing both mutants was named pKL246. These results were based on growth phenotypes on agar. Only two of these plasmids (pKL245 and pKL246) complemented the same mutants for growth with AEPn as a sole P source in broth cultures, suggesting that the other one (pKL242) was unstable and had lost a segment required for complementation. Since it was later shown that the latter lacked DNA corresponding to the *S. typhimurium phn* locus, this plasmid served as a negative control in subsequent experiments.

The only genes for the phosphonatase pathway that have been studied previously are from *Enterobacter aerogenes* (24). It was therefore of interest to test them for hybridization to *S. typhimurium* DNA as well as to test our complementing plasmids for hybridization to each other. Figure 2A shows that a plasmid carrying genes for the *Enterobacter aerogenes* phosphonatase pathway (pEG5166-KL38) hybridizes to *S. typhimurium* chromosomal DNA. Because four different enzymes produced similar levels of hybridization, the homologous sequences must include *phn* sequences in common between these bacteria. There may also be homologous sequences due to adjacent genes in common, although the chromosomal insert of this plasmid appears to be largely *phn* sequences.

We also tested plasmids carrying *S. typhimurium phn* sequences for hybridization. As controls, we examined plasmids carrying genes for the C-P lyase pathways of *E. coli* and *Enterobacter aerogenes*, pBW120 and pKL124, respectively. Figure 2B shows an ethidium bromide-stained gel of plasmid DNAs cut with as many as four enzymes. Figure 2C shows those DNA fragments hybridizable to the vector pEG5294. Figure 2D to F show that both plasmids carrying *S. typhimurium phn* sequences (pKL245 and pKL246) as well as one carrying genes for the phosphonatase pathway of *Enterobacter aerogenes* (pKL38) contain hybridizable fragments that are clearly not attributable to vector sequences. In addition, the plasmids carrying *S. typhimurium phn* sequences contain the same chromosomal region, and it is these sequences that are homologous to *Enterobacter aerogenes phn* sequences for the phosphonatase pathway. Figure 2D to F show that no additional fragment of a control plasmid lacking *phn* sequences (pKL242) is homologous to sequences carried by the *S. typhimurium* and *Enterobacter aerogenes* phosphonatase plasmids. Comparison of these panels with Figure 2C reveals that those sequences in common

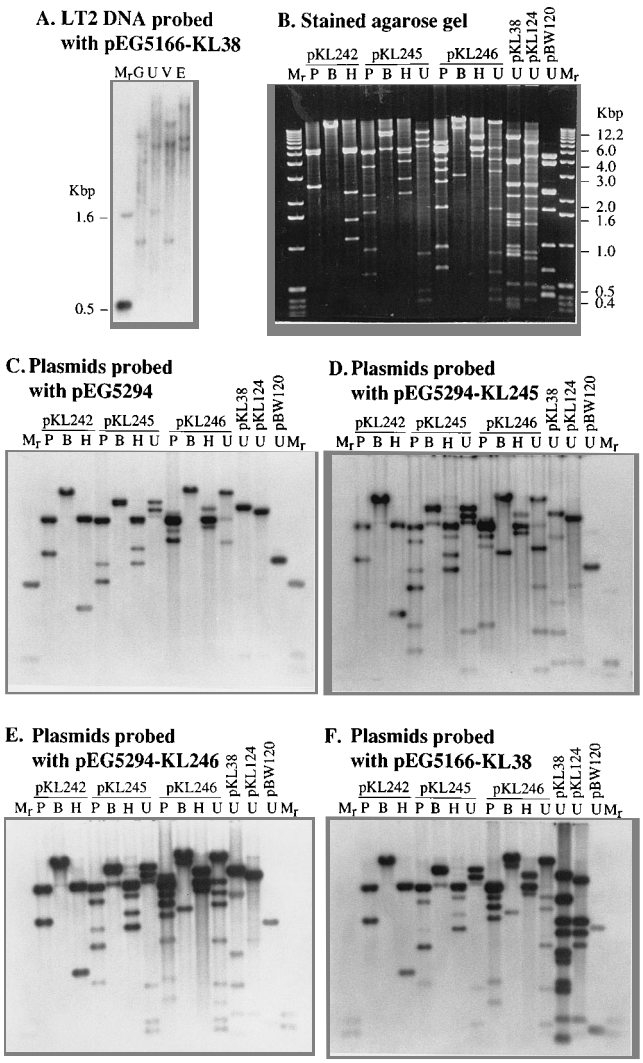

FIG. 2. DNA hybridization of *S. typhimurium phn* sequences. Chromosomal and plasmid DNAs were digested with restriction enzymes, separated with 0.7% agarose gels, and tested for hybridization with ^{32}P-labeled probes as described previously (51). pKL38 is a derivative of the mini-Mu vector pEG5166 carrying genes for the phosphonatase pathway of *Enterobacter aerogenes*; pKL38 and pEG5166-KL38 refer to the same plasmid. pKL245 and pKL246 are derivatives of the mini-Mu vector pEG5294 carrying genes for the phosphonatase pathway of *S. typhimurium* and are the same as pEG5294-KL245 and pEG5294-KL246, respectively. Abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; U, *Pvu*II.

between plasmids encoding the phosphonatase pathway and plasmids encoding the C-P lyase pathway are attributable to vector sequences. In agreement, no evidence for DNA homology was found when plasmids encoding the phosphonatase and C-P lyase pathways from *Enterobacter aerogenes* were compared (24).

The ability to break down AEPn by the phosphonatase pathway of *Enterobacter aerogenes* is under Pho regulon control (24). This was shown by the inability of a plasmid encoding the phosphonatase pathway of *Enterobacter aerogenes*(pKL38) to yield $AEPn^+$ transformants of an *E. coli phoB* mutant, which is defective in the Pho regulon transcriptional activator. To determine whether *S. typhimurium* genes for the phosphonatase pathway are under Pho regulon control, we transformed the Δ*phoBR* mutant BW14649 (a Mu-1 lysogen) with pKL246.

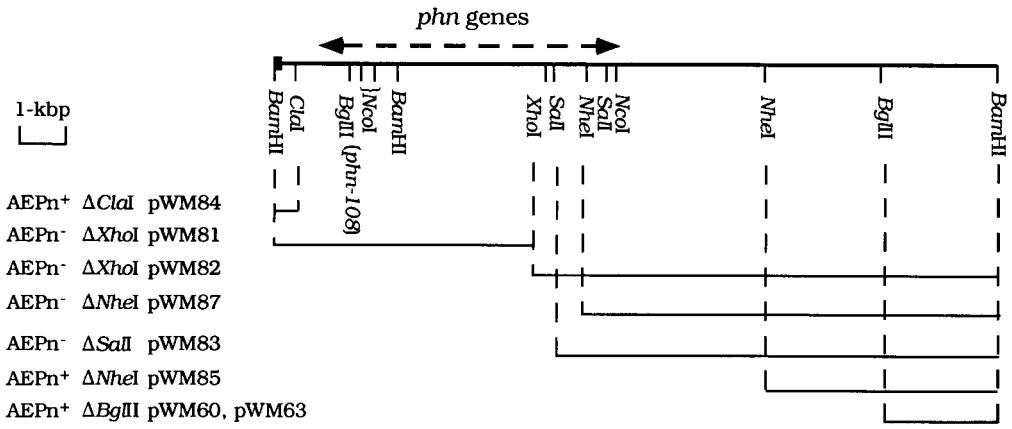

FIG. 3. Restriction map of the *S. typhimurium phn* locus. The 14.9-kbp chromosomal fragment for genes of the phosphonatase pathway in *S. typhimurium* is shown. Plasmids carrying this fragment in opposite orientations in the polylinker of a cloning vector were named pWM67 and pWM68. These plasmids were used to generate deletions between sites within the insert and various adjacent polylinker sites. The deleted plasmids were verified by restriction mapping and tested for complementation by transformation of various *pir*$^+$ *E. coli phn* mutants, as described in Materials and Methods. AEPn$^-$ refers to the inability of the resultant plasmid to complement a Δ(*phnCDEFGHIJKLMNOP*) mutant. The thickened line on the far left end of the insert corresponds to Mu DNA from the mini-Mu vector. The *Bgl*II site nearer the left side corresponds to the *phn-108* allele. pWM81, pWM84, pWM85, and pWM87 are derivatives of pWM67, and pWM82 and pWM83 are derivatives of pWM68. pWM60 and pWM63 are derivatives of pEG5294-MK10 that resulted from partial *Bgl*II digestion and ligation (see Materials and Methods).

Because these transformants were AEPn$^-$, the expression of genes for the *S. typhimurium* phosphonatase pathway also appears to be under Pho regulon control. We also showed that mutations of the *S. typhimurium phoP* and *phoN* loci are without effect on utilization of AEPn. In particular, we showed that the *phoP53*::Tn*10* mutation of MS509 and the *phoN51*::Tn*10* mutation of TT13206 (Table 1) are without an effect on AEPn utilization by transferring these mutations into MS1868 (data not shown). We did this in part because *phoN* (at 94.2 min) of *S. typhimurium* lies in the vicinity of the *phn* locus (at 92.8 min) of *E. coli.*

Subcloning and mutagenesis of the *S. typhimurium phn* locus. We showed that the *phn* genes correspond to an approximately 7.2-kbp region on the left side of the insert shown in Fig. 3. Because of the instability of mini-Mu plasmids, we subcloned various fragments into another vector (see Materials and Methods). In particular, we subcloned a 14.9-kbp partial *Bam*HI fragment that is capable of complementing an *E. coli* mutant deleted of the entire *phnC*-to-*phnP* locus. We made several smaller derivatives of these plasmids and tested them for complementation. The results summarized in Fig. 3 are consistent with a 7.2-kbp region containing the entire *S. typhimurium phn* locus. All transposon-induced mutations resulting in an AEPn$^-$ phenotype were also shown to lie within the same region (data not shown).

We showed that sequences on the left end of the 7.2-kbp region encode catalysis functions for the phosphonatase pathway and that genes on the right end are required for Pn transport. The 7.2-kbp segment contains an internal *Bam*HI site. Plasmids carrying DNA to the left of this site are capable of complementing a Δ(*phnHIJKLPMNOP*) mutant (C-P lyase defective) but not a Δ(*phnCDEFGHIJKLMNOP*) mutant (Pn transport and C-P lyase defective). Therefore, sequences to the left of the *Bam*HI site encode enzymes for breakdown of AEPn. Mini-Mu plasmids capable of complementing only a Δ(*phnHIJKLPMNOP*) mutant were also identified (see Materials and Methods). These plasmids also carried DNA to the left of the *Bam*HI site. Conversely, sequences to the right of this *Bam*HI site probably encode a transporter for the phosphonatase pathway. No plasmid capable of complementing an *E. coli* mutant that is defective only in transport for the C-P lyase pathway was identified, however.

We also showed that the 7.2-kbp region appears to be transcribed as two (or more) divergent genes (or operons). We showed this by interrupting the *phn* region with a *uidA* cassette and by transposon mutagenesis. We inserted a promoterless *uidA* cassette in the *Bgl*II site (the *phn-108* allele) near the left end of the insert. Those plasmids expressing *uidA* have the reporter gene oriented leftward. Therefore, this region is transcribed from right to left (as shown in Fig. 3). Several *phoA* or *lacZ* fusions resulting from transposon mutagenesis express the corresponding reporter gene. All actively transcribed fusions due to an insertion to the left of the *Bam*HI site are oriented leftward, while all actively transcribed fusions due to an insertion to the right of the *Bam*HI site are oriented rightward (data not shown).

Cloning and mutagenesis of the *S. typhimurium phoB* locus. We found that genes for the *S. typhimurium* phosphonatase pathway, like those from *Enterobacter aerogenes* (24), are under Pho regulon control. This was initially shown by transformation of an *E. coli phoB* mutant with our AEPn$^+$ plasmids. These transformants were unable to grow with AEPn as a sole P source. A negative growth phenotype is expected because PhoB is the transcriptional activator of Pho regulon promoters. To verify that these *phn* genes are under Pho regulon control in *S. typhimurium*, we cloned the *phoB* locus of *S. typhimurium* by complementation of an *E. coli phoB* mutant (see Materials and Methods). Of 12 presumptive *phoB*$^+$ plasmids, two also carried the nearby *proC*$^+$ gene (as expected by analogy to *E. coli*). These plasmids were used to construct *phoB* deletion and insertion mutations by use of a *cat* cassette or by transposon mutagenesis. Each of the resulting *phoB* mutations was then recombined onto the chromosome by allele replacement.

Three different *phoB* mutations abolished the ability of *S. typhimurium* to use AEPn as a sole P source. Growth of these *phoB* mutants on AEPn was restored by complementation with an *E. coli* K-12 *phoB*$^+$ plasmid. In addition, we verified that the *S. typhimurium phoB* mutants have an allele replacement by DNA hybridization (Fig. 4). We also proved that the *S. typhimurium phoB* gene was cloned by showing that the same chro-

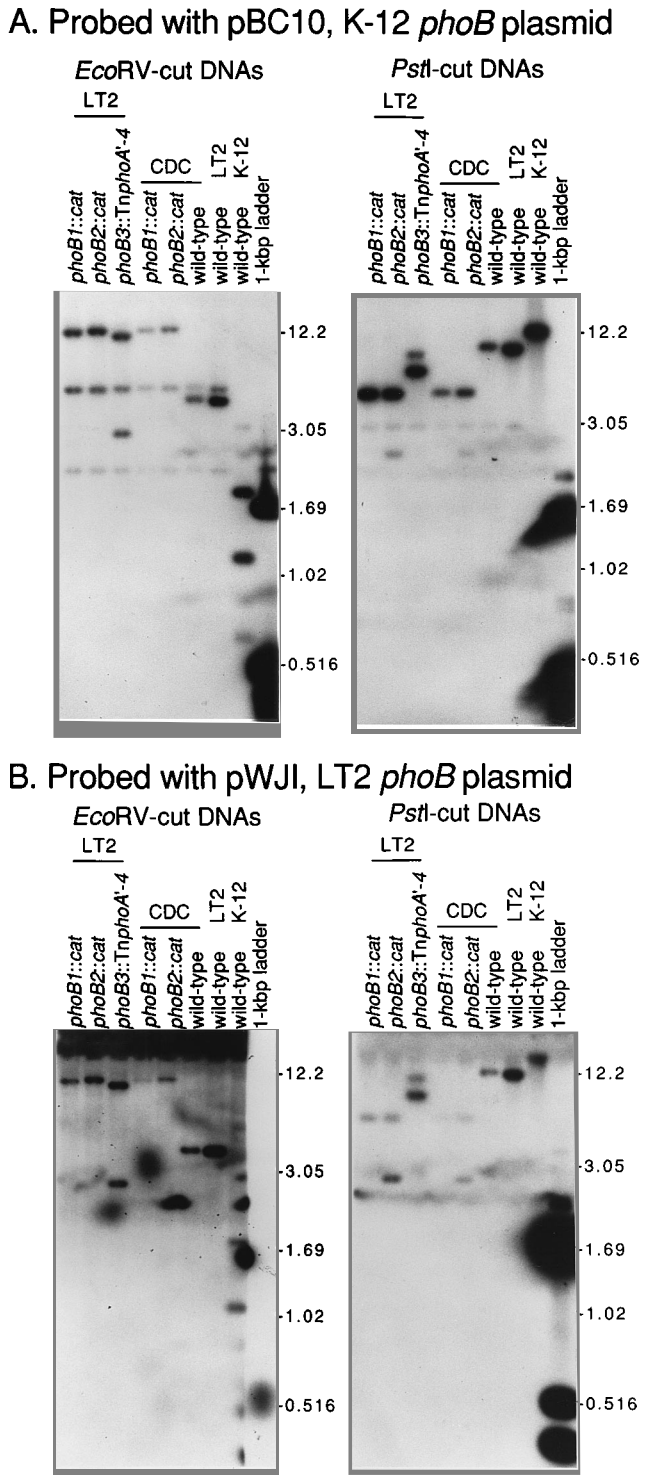

FIG 4. DNA hybridization of *S. typhimurium phoB* locus. Chromosomal DNAs were digested with *Eco*RV and *Pst*I, separated with a 0.7% agarose gel, and tested for hybridization with ^{32}P-labeled DNA of an *E. coli* K-12 *phoB*$^+$ plasmid (pBC10) or an *S. typhimurium* LT2 *phoB*$^+$ plasmid (pWJ1), as described previously (51). DNAs from the following strains were examined: the *S. typhimurium* LT2 derivatives TBW19812 (Δ*phoB1*::*cat*), TBW19813 (*phoB2*::*cat*), and TBW19815 (*phoB3*::Tn*phoA′-4*); the *S. typhimurium* CDC 6516-60 derivatives TBW19931 (Δ*phoB1*::*cat*) and TBW19932 (*phoB2*::*cat*); *S. typhimurium* CDC 6516-60; *S. typhimurium* LT2 strain MS1868; and *E. coli* K-12 strain BW13711. The numerals on the right side of each panel are the sizes in kilobase pairs of molecular mass markers.

mosomal fragments hybridize when probed with plasmid DNA carrying *E. coli* K-12 or *S. typhimurium* LT2 *phoB* DNA (compare Fig. 4A and Fig. 4B).

The results described above indicate that the genes for the phosphonatase pathway are members of the *S. typhimurium* Pho regulon. We therefore examined the expression of the Pho regulon in *S. typhimurium*. In *E. coli*, *phoA* is a convenient reporter of the Pho regulon; however, *phoA* is absent in *S. typhimurium*. Hence, we introduced a *phoA-lacZ* transcriptional fusion into *S. typhimurium*. To do this, we transferred the λRZ*5phoA-lacZ* phage from a lysogen of an *E. coli* HfrH strain into *S. typhimurium* by conjugation as described in Materials and Methods. The resulting strain showed a phosphate-starvation-inducible Lac$^+$ phenotype, and it had λRZ*5phoA-lacZ* phage integrated in *att*λ of *S. typhimurium* (see Materials and Methods).

The expression of the *E. coli* Pho regulon is inhibited when P_i is in excess and activated under conditions of P_i limitation. Inhibition requires an intact Pst system, and activation requires PhoB. As expected, the *phoA-lacZ* fusion was expressed at a low basal level in the presence of excess P_i; its expression was activated about 200-fold under conditions of P_i limitation (Table 2). Inhibition was abolished by a *pst*::Tn*10* mutation which resulted in high-level activation independent of the P_i level. Activation of *phoA-lacZ* expression resulting from P_i limitation or the *pst*::Tn*10* mutation was abolished by the Δ*phoB1*::*cat* or *phoB2*::*cat* mutations. These results confirm that Pho regulon control is fundamentally similar in *E. coli* and *S. typhimurium*.

Mapping of the *S. typhimurium phn* and *phoB* loci. We mapped the *phn* locus for the phosphonatase pathway near 10 min on the *S. typhimurium* chromosome. In contrast, the *phn* locus for the C-P lyase pathway of *E. coli* lies near 93 min (51). Three lines of evidence are consistent with a location in the vicinity of 10 min. A plasmid carrying *S. typhimurium phn* sequences (pKL246, a derivative of the mini-Mu vector pEG5294) showed higher levels of hybridization to DNAs from lysates of Mu*d*-P22 prophages (7) corresponding to this region. However, this plasmid showed the highest levels of hybridization to DNAs corresponding to the region near 60 min. Apparently, the 60-min region contains different sequences that are homologous to this plasmid. For example, DNAs prepared from Mu*d*-P22 lysates for the 60-min region may contain additional Mu sequences that are also present in the mini-Mu vector pEG5294. Importantly, lysates of Mu*d*-P22 prophages corresponding to the 10-min region but not to the 60-min region yielded AEPn$^+$ transductants of TBW18330 (SL1657 *phn-103*::Tn*phoA′-1*). In addition, by using P22 lysates prepared on strains with a variety of Tn*10* insertions in the 10-min and 60-min regions, we showed several chromosomal *phn* mutations to be linked to the 10-min region but not to the 60-min region (data not shown).

We showed the gene order of the 9- to 10-min chromosomal region of *S. typhimurium* to be *proC phoB phn* (Fig. 5). The corresponding gene order of *E. coli* is *phoA proC phoB* (4). By testing several strains with mutations in this region, we discovered that the *S. typhimurium phn* locus corresponds to a locus of *S. typhimurium* previously called *psiC* (14), a phosphate-starvation-inducible locus for which no function had been previously identified. We also found that the *zba-34*::Tn*10* insertion (which is closely linked to *psiC*) is located within the *phn* locus. Strains carrying either of these mutations are AEPn$^-$, and the AEPn$^-$ phenotype is inseparable from the *zba-34*::Tn*10* and *psiC17*::*lacZ* (Mu d1-1734) insertions in P22 crosses. Of more than 200 Tet^r or Kan^r transductants of an AEPn$^+$ recipient, all were AEPn$^-$ (data not shown). We there-

TABLE 2. Pho regulon control of *phoA* expression in *S. typhimurium* LT2

Strain	Relevant genotype[a]	β-Galactosidase sp act[b] on[c]:	
		Excess P_i	Limited P_i
TBW13740	λRZ5*phoA-lacZ*$_{DK110}$	20.9 ± 0.6	4,205 ± 195
TBW13842	λRZ5*phoA-lacZ*$_{DK110}$ *pst-4*::Tn*10*	3,841 ± 217	3,486 ± 26
TBW19866	λRZ5*phoA-lacZ*$_{DK110}$ Δ*phoB1*::*cat*	6.9 ± 0.6	11.2 ± 0.5
TBW19867	λRZ5*phoA-lacZ*$_{DK110}$ *phoB2*::*cat*	8.7 ± 0.6	11.9 ± 0.7
TBW19868	λRZ5*phoA-lacZ*$_{DK110}$ Δ*phoB1*::*cat pst-4*::Tn*10*	9.1 ± 0.3	9.7 ± 0.5
TBW19869	λRZ5*phoA-lacZ*$_{DK110}$ *phoB2*::*cat pst-4*::Tn*10*	7.5 ± 0.7	10.6 ± 0.5

[a] All strains are otherwise isogenic derivatives of SL1657 (Table 1).
[b] Specific activity (sp act) values are nanomoles of *o*-nitrophenol made per minute per cell culture optical density at 420 nm.
[c] Cells were assayed following growth with excess P_i or limited P_i as described in Materials and Methods. Strains were grown and assayed in triplicate. Values are means ± standard deviations.

fore renamed the *psiC17*::*lacZ* (Mu d1-1734) and *zba-34*::Tn*10* insertions as the *phn-128* and *phn-129* alleles, respectively.

The linkage data indicate the order of these insertions to be *phn-129*::Tn*10* *phn-128*::*lacZ* (Mu d1-1734) *phn-108*::*uidA*, in a clockwise order (Fig. 5). The genetic linkages between these insertions are consistent with the *phn* locus spanning about 7 kb, since individual insertions are estimated to be at least 2 kb apart (data not shown). We were also able to infer the orientation of the *lacZ* and *uidA* transcriptional fusions. As mentioned above, the *phn-108*::*uidA* insertion lies near one end of the *phn* locus. In addition, all transposon Tn*phoA*, Tn*phoA′-1*, and Tn*phoA′-4* insertions (see Materials and Methods) resulting in expression of the respective reporter genes that are located upstream of the *phn-108*::*uidA* insertion site are oriented in the opposite direction. Yet none of the insertions illustrated in Fig. 5 has a polar effect on the *lacZ* or *uidA* fusion. The *phn-129*::Tn*10* and *phn-128*::*lacZ* (Mu d1-1734) mutations have no effect on expression of the *phn-108*::*uidA* fusion, and the *phn-129*::Tn*10* and *phn-108*::*uidA* mutations have no effect on expression of the *phn-128*::*lacZ* (Mu d1-1734) fusion (data not shown). Together, these results are consistent with clockwise transcription of the *phn-108*::*uidA* fusion and counterclockwise transcription of the *phn-128*::*lacZ* (Mu d1-1734) fusion, as shown in Fig. 5.

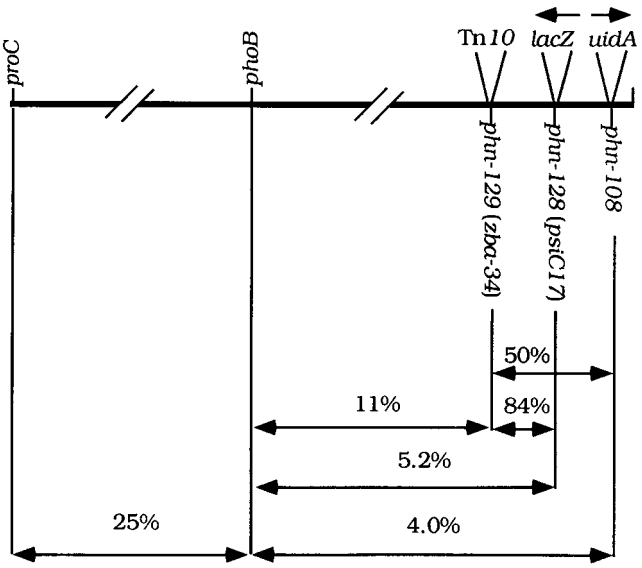

FIG. 5. Gene organization near the *S. typhimurium phoB* and *phn* loci. Genetic linkages are summarized from a series of P22 crosses. Linkages were calculated as described elsewhere (42), by taking into account the sizes of insertion elements. Individual transductions involved two- or three-factor crosses. Pro$^+$ transductants of TT15230 (*proC693*::MudP) were selected with P22 grown on TBW19800 (Δ*phoB1*::*cat*); Tet^r transductants of TBW20169 (Δ*phoB1*::*cat phn-108*::*uidA2-aphA*) and TBW20170 [Δ*phoB1*::*cat phn-128*::*lacZ* (Mu d1-1734)] were selected with P22 grown on JF562 (*phn-129*::Tn*10*); Cm^r transductants of TBW20122 (*phn-108*::*uidA2-aphA*), TBW20123 [*phn-128*::*lacZ* (Mu d1-1734)], TBW20124 (*phn-129*::Tn*10*), and TBW20180 [*phn-129*::Tn*10 phn-128*::*lacZ* (Mu d1-1734)] were selected with P22 grown on TBW19800 (Δ*phoB1*::*cat*); and Tet^r transductants of TBW20903 (*phn-108*::*uidA2-aadA*) and TBW20904 (*phn-108*::*uidA2-cat*) were selected with P22 grown on TBW20893 [*phn-129*::Tn*10 phn-128*::*lacZ* (Mu d1-1734)]. In most cases, about 200 Pro$^+$ or antibiotic-resistant transductants were purified at least once prior to scoring of relevant phenotypes.

By testing these insertions for complementation, the *phn-108*::*uidA* mutation was shown to be complemented by a plasmid carrying only catalysis genes, and the *phn-129*::Tn*10* and *phn-128*::*lacZ* (Mu d1-1734) mutations were shown not to be complemented. Therefore, the *phn-108*::*uidA* insertion probably lies in a gene required for catalysis, and the *phn-129*::Tn*10* and *phn-128*::*lacZ* (Mu d1-1734) mutations may lie in genes required for transport. We were unable to show definitively that the latter lie in a transport gene(s) since we were unable to find a plasmid capable of complementing a mutant defective only in transport, as mentioned above. The alternative possibility that the *phn-128* and *phn-129* insertions are in a gene(s) for a new transcriptional activator is unlikely because the expression of all reporter fusions to these *phn* genes appears to be activated by PhoB (see below and data not shown).

Regulation of the *S. typhimurium phn* locus. By comparing expression of the *phn-108*::*uidA* and the *phoA-lacZ* fusions in the same strain, we proved the *phn* locus to be regulated as a member of the Pho regulon (Table 3). A ca. ninefold activation of the *phn*::*uidA* fusion and a 700-fold activation of the *phoA-lacZ* fusion under conditions of P_i limitation were found. Activation of both fusions is abolished by a *phoB* mutation and is rendered constitutive by a *pst* mutation. As expected, a *phoB* mutation is epistatic to a *pst* mutation. Hence, the *phn-108*::*uidA* fusion appears to be expressed from a promoter under Pho regulon control. In addition, a ca. twofold effect on the activation of the *phoA-lacZ* fusion is observed even in the presence of a *pst* mutation. This effect appears to be *phoB* independent. Although the effect is small, it appears to be significant. Its basis is not understood, however.

Testing for an effect of *phoB* and *phn* mutations on pathogenicity. Our discovery that *S. typhimurium* encodes a pathway for Pn degradation that is absent in *E. coli* led us to consider a role for this pathway in the virulence of *S. typhimurium*. Because Pn in nature exist primarily in the form of phosphonolipids, enzymes of this pathway may aid in membrane destruction by invasive bacteria. We therefore compared the virulence of *S. typhimurium* CDC 6516-60 with that of its isogenic deriv-

TABLE 3. Pho regulon control of *phn*::*uidA* and *phoA-lacZ* fusions in *S. typhimurium* LT2

Relevant genotype[a]	β-Glucuronidase sp act[b] on[c]:		β-Galactosidase sp act[b] on[c]:	
	0.06% Glucose–2 mM P_i	0.4% Glucose–0.1 mM P_i	0.6% Glucose–2 mM P_i	0.4% Glucose–0.1 mM P_i
Wild type	0.013 ± 0.002	0.120 ± 0.002	31.1 ± 1.1	21,700 ± 1,460
Δ*phoB1*	0.014 ± 0.001	0.013 ± 0.001	26.4 ± 1.8	40.5 ± 7.0
pst-4	0.120 ± 0.005	0.140 ± 0.005	9,230 ± 145	21,200 ± 1,290
Δ*phoB1 pst-4*	0.012 ± 0.006	0.010 ± 0.006	35.2 ± 3.5	67.1 ± 6.1

[a] All strains carry both *phn-108*::*uidA2-aph* and λRZ5*phoA-lacZ*$_{DK110}$ and are otherwise wild type, Δ*phoB1*::*cat*, or *pst-4*::Tn*10*, as indicated. All are isogenic derivatives of the SL1657 (Table 1) exconjugant carrying λRZ5*phoA-lacZ*$_{DK110}$ named TBW13740 (see Materials and Methods). Additional mutations were introduced by P22 transduction. Strains assayed included TBW20912, TBW20913, TBW20914, and TBW20915, respectively.
[b] Values are nanomoles of *o*-nitrophenol made per minute per milligram of protein (means ± standard deviations; see Materials and Methods).
[c] Cells were grown and assayed as described in Table 2, footnote *c*.

atives TBW19931 (CDC 6516-60 Δ*phoB1*::*cat*) and TBW19989 (CDC 6516-60 *phn-116*::Tn*phoA′-1*) in competition infections of mice (37). Following oral or intraperitoneal infection at different ratios and dosages, no effect on virulence was observed. The ratios of mutant to wild-type bacteria in spleens of infected mice were similar to those at the time of infection (data not shown). In these experiments, bacteria were pregrown in Luria broth, and infections were carried out after the bacteria were suspended in bicarbonate buffer (44).

Expression of other *S. typhimurium psi* loci. As mentioned above, the *phn* locus corresponds to a locus previously called *psiC*. Three other phosphate-starvation-inducible loci (*psiA*, *psiB*, and *psiD*) have also been identified previously in *S. typhimurium* (14). It was therefore of interest to compare the levels of expression of these *psi* loci in otherwise isogenic wild-type, *phoB*, and *pst* strains. In brief, we showed that the *psiB12*::*lacZ* and *phn-128* (*psiC17*)::*lacZ* fusions are regulated like members of the Pho regulon (Table 4). Expression of the *psiB12*::*lacZ* and *phn-128*::*lacZ* fusions is activated ca. 4.5-fold and 30-fold under conditions of P_i limitation, respectively. Activation of these fusions is abolished by a *phoB* mutation and is rendered constitutive by a *pst* mutation. An unlinked locus named *psiR* was also identified in which a mutation led to constitutive expression of *psiC*. Accordingly, the *psiR* locus (14) of *S. typhimurium* probably corresponds to the *pstSCAB-phoU* operon of *E. coli* (45). The *psiR*::Tn*10* mutation leads to constitutive expression of the *phn-128*::*lacZ* fusion and lies in the vicinity of the *E. coli pstSCAB-phoU* operon.

In contrast, the *psiA9*::*lacZ* and *psiD19*::*lacZ* fusions are not regulated like members of the Pho regulon. Expression of the *psiA9*::*lacZ* fusion is unaffected by P_i limitation, a *phoB* mutation, or a *pst* mutation (Table 4). The expression of the *psiA9*::*lacZ* fusion was previously shown to be activated ca. threefold under conditions of P_i limitation in a different strain background (JF511 in Table 1). A different pattern is seen for the expression of the *psiD19*::*lacZ* fusion. Although the expression of this fusion is activated ca. twofold by P_i limitation, this activation appears to be independent of *phoB*. Yet the *psiD19*::*lacZ* fusion is expressed at a higher level in a *pst* mutant. In these ways, the *psiD19*::*lacZ* fusion behaves like some *psi* fusions of *E. coli* that are not members of the Pho regulon (53).

DISCUSSION

It has been known for a long time that *Bacillus cereus* and *P. aeruginosa* are capable of AEPn breakdown by the phosphonatase pathway (21, 39), although genes for this pathway from these bacteria have yet to be characterized. We had previously reported the first cloning of genes for this pathway from *Enterobacter aerogenes* (24). However, *Enterobacter aerogenes* carries in addition genes for the C-P lyase pathway that are homologous to those of *E. coli* (51). The presence of two Pn degradation pathways in *Enterobacter aerogenes* precluded detailed studies of Pn breakdown in this bacterium. Because of similarities between *E. coli* Δ*phn* mutants carrying plasmids for the *Enterobacter aerogenes* phosphonatase pathway and *S. typhimurium*, we suspected that *S. typhimurium* may have only the phosphonatase pathway. Plasmids carrying genes for the phosphonatase pathway from *Enterobacter aerogenes* allowed the growth of *E. coli* Δ*phn* mutants only on AEPn, and on no

TABLE 4. Effects of P_i limitation on expression of *psiA*, *psiB*, *psiC*(*phn*), and *psiD*::*lacZ* fusions

Strain[a]	Fusion	Relevant genotype	β-Galactosidase sp act[b] on[c]:	
			0.06% Glucose–2 mM P_i	0.4% Glucose–0.1 mM P_i
TBW20119	*psiA9*::*lacZ* (Mu d1-1734)	Wild type	84.1 ± 1.2	64.2 ± 0.9
TBW20120	*psiA9*::*lacZ* (Mu d1-1734)	Δ*phoB1*::*cat*	79.0 ± 3.2	68.2 ± 1.0
TBW20121	*psiA9*::*lacZ* (Mu d1-1734)	*pst-4*::Tn*10*	260 ± 21	217 ± 9
TBW19970	*psiB12*::*lacZ* (Mu d1-1734)	Wild type	0.70 ± 0.02	3.2 ± 0.2
TBW19972	*psiB12*::*lacZ* (Mu d1-1734)	Δ*phoB1*::*cat*	0.44 ± 0.01	0.89 ± 0.04
TBW19974	*pstB12*::*lacZ* (Mu d1-1734)	*pst4*::Tn*10*	7.2 ± 0.2	5.6 ± 0.1
TBW19971	*psiC17*::*lacZ* (Mu d1-1734)	Wild type	0.42 ± 0.01	12.5 ± 0.7
TBW19973	*psiC17*::*lacZ* (Mu d1-1734)	Δ*phoB1*::*cat*	0.40 ± 0.02	0.33 ± 0.02
TBW19975	*psiC17*::*lacZ* (Mu d1-1734)	*pst-4*::Tn*10*	25.0 ± 0.9	21.1 ± 0.1
TBW20020	*psiD19*::*lacZ* (Mu d1-1734)	Wild type	91.5 ± 2.3	226 ± 9
TBW20021	*psiD19*::*lacZ* (Mu d1-1734)	Δ*phoB1*::*cat*	102 ± 3	176 ± 3
TBW20022	*psiD19*::*lacZ* (Mu d1-1734)	*pst-4*::Tn*10*	226 ± 1	384 ± 9

[a] All strains are isogenic derivatives of MS1868 (Table 1) that were made by P22 transduction.
[b] Values are nanomoles of *o*-nitrophenol made per minute per cell culture optical density at 420 nm (means ± standard deviations).
[c] Strains were grown and assayed as described in Table 2, footnote *c*.

other Pn, as a sole P source. Likewise, *S. typhimurium* exhibits the same Pn phenotype with respect to growth with AEPn as a sole P source (51).

We therefore cloned genes for AEPn utilization from *S. typhimurium* by complementation of *E. coli* Δ*phn* mutants. We showed that these genes encode proteins for the phosphonatase pathway as they correspond to homologs of *Enterobacter aerogenes*. In agreement, genes for AEPn utilization from *S. typhimurium* are not homologous to those for the C-P lyase pathway from *E. coli* or *Enterobacter aerogenes*. We isolated two kinds of complementing plasmids. One kind probably encodes proteins for both transport and catalysis of C-P bond cleavage. These plasmids are capable of complementing an *E. coli* Δ*phn* mutant lacking genes for both Pn transport and catalysis. The other kind probably encodes only proteins for catalysis. These are capable of complementing only an *E. coli* Δ*phn* mutant lacking genes for catalysis but containing genes for Pn transport. In addition, we attempted to identify a gene(s) for Pn transport by complementation of an *E. coli* Δ*phn* mutant defective only in transport. However, none was found. This failure to demonstrate directly the presence of an AEPn transporter is an enigma. It appears that genes for the AEPn transporter are dispersed on the complementing fragment or that the AEPn transporter for the phosphonatase pathway is unable to transport AEPn for utilization by the C-P lyase pathway.

Our smallest plasmid capable of complementing mutants lacking both Pn transport and catalysis functions has an insert greater than 7 kb in length; our smallest plasmid capable of complementing mutants lacking only catalysis functions has a ca. 2.7-kb insert. Results based on transposon mutagenesis of these plasmids are consistent with a ca. 7.2-kb segment encoding genes for transport and catalysis. Accordingly, genes for AEPn transport may constitute ca. 4 to 5 kb, and genes for catalysis may constitute at least 2 kb. These data suggest that the *S. typhimurium* AEPn transporter is a multicomponent transporter similar to the binding protein-dependent Pn transporter for the C-P lyase pathway of *E. coli*. These data are also consistent with two enzymes being required for catalysis, an AEPn-specific transaminase and a phosphonatase (Fig. 1B). Proof of this must await more detailed characterization of these genes and their gene products, however. Whether the *S. typhimurium phn* locus also encodes a regulatory protein(s) similar to those of the *E. coli phn* locus is unknown.

Like genes for the C-P lyase pathway of *E. coli*, genes for both the C-P lyase and the phosphonatase pathways of *Enterobacter aerogenes* appear to be under Pho regulon control (24). Here we showed that genes for the *S. typhimurium* phosphonatase pathway are also under Pho regulon control. The expression of the *E. coli* Pho regulon is activated under conditions of P_i limitation, a process requiring PhoB. We showed that plasmids carrying genes for the *S. typhimurium* phosphonatase pathway are unable to complement an *E. coli phoB* mutant for AEPn utilization. We verified this by cloning *phoB* from *S. typhimurium* and constructing *S. typhimurium phoB* mutants by allele replacement. These *phoB* mutants are unable to use AEPn as a sole P source. In addition, we showed that *uidA* and *lacZ* transcriptional fusions to two separate regions of the *phn* locus are under Pho regulon control in *S. typhimurium*. The expression of these fusions is activated under conditions of P_i limitation, and activation of them is abolished in *S. typhimurium phoB* mutants. The expression of the *E. coli* Pho regulon is inhibited by P_i only in the presence of an intact phosphate-specific transport (Pst) system. Likewise, *phn*::*uidA* and *phn*::*lacZ* transcriptional fusions are expressed at high constitutive levels in an *S. typhimurium pst* mutant.

Furthermore, we confirmed the *S. typhimurium* Pho regulon to be regulated in the same way as the *E. coli* Pho regulon. We did this by constructing an *S. typhimurium* derivative carrying an *E. coli phoA-lacZ* transcriptional fusion in a single copy. As expected, activation of the *phoA-lacZ* fusion is abolished in *S. typhimurium phoB* mutants, and expression is rendered constitutive in an *S. typhimurium pst* mutant. Although it had been shown previously that *phoA* of *E. coli* is similarly regulated in *S. typhimurium*, those studies involved introduction of an F′ carrying *phoA* into *S. typhimurium* (43). Because the F′ used in those studies also carried the *E. coli phoBR* operon, they were inconclusive in regard to *phoB*. Those studies established only *phoA* expression to be inhibited by P_i in *S. typhimurium*, a process requiring all components encoded by the unlinked *pstSCAB-phoU* operon in *E. coli*. In addition, *S. typhimurium* has been shown to carry *phoR* by complementation of an *E. coli* F′ carrying a *phoR* mutation (56).

The *E. coli phn* gene cluster lies near 93 min and has been shown to correspond to the *psiD* locus (46, 51). In contrast, the *S. typhimurium phn* locus lies near 10 min (this study), at a locus previously called *psiC* (14). Although the *S. typhimurium phn* locus lies in the vicinity of the *phoBR* operon, the distance between them is too great to indicate any significance (Fig. 5). Because *phoN* (encoding a nonspecific acid phosphatase) of *S. typhimurium* lies near 94 min, we tested for an effect of *phoN* on AEPn utilization; however, no effect was observed. There was also no effect on AEPn utilization due to *phoP* (encoding a transcriptional activator of *phoN* and coregulated genes). Accordingly, there is no evidence for a regulatory connection between the Pho regulon and the PhoP-PhoQ two-component regulatory system in *S. typhimurium* or in *E. coli* (unpublished data).

We considered a possible role of the *S. typhimurium phn* locus in pathogenicity, largely because AEPn is found primarily in phosphonolipids. Hence, we tested for an effect on virulence due to *phoB* and *phn* mutations; however, no effect was observed. It should be noted that these experiments were carried out with *S. typhimurium* pregrown in the presence of excess P_i. The possibility that an effect may result from infection by P_i-limited bacteria has not been ruled out.

The *E. coli* Pho regulon consists of at least 31 coregulated genes, including the *phoA-psiF* operon, the *phoBR* operon, *phoE*, *phoH*, the 14-gene *phnC*-to-*phnP* operon, the *pstSCAB-phoU* operon, the *ugpBAECQ* operon, and *psiE* (49, 50). Sixteen of these are absent in *S. typhimurium*. It has long been known that *phoA* is absent; *psiF* and the *phnC*-to-*phnP* operon are also absent, while several other Pho regulon genes appear to be present in *S. typhimurium*. Both *phoE* (5) and the *ugp*-encoded uptake system (16) are present. The *phoBR* and *pstSCAB-phoU* operons are also present. Whether the *ugp*, *phoBR*, and *pstSCAB-phoU* operons share identical gene arrangements in *E. coli* and *S. typhimurium* has not been established, however. In addition, the *psiB* locus of *S. typhimurium* appears to be a member of the Pho regulon, but its function is unknown. The *psiR* locus of *S. typhimurium* probably corresponds to the *pstSCAB-phoU* operon. On the basis of its DNA sequence (31), the *S. typhimurium phn* locus appears to encode seven genes that we have named *phnR* to *phnX* (manuscript in preparation). Thus, the *S. typhimurium* Pho regulon appears to consist of at least 21 genes.

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