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Accumulation of Intermediates of the Carbon-Phosphorus Lyase Pathway for Phosphonate Degradation in *phn* Mutants of *Escherichia coli*[▽]

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The catabolism of phosphonic acids occurs in *Escherichia coli* by the carbon-phosphorus lyase pathway, which is governed by the 14-cistron *phn* operon. Here, several compounds are shown to accumulate in strains of *E. coli* with genetic blocks in various *phn* cistrons when the strains are fed with phosphonate.

Phosphonates (Pn), which contain the carbon-phosphorus bond, are quite abundant in nature, primarily as components of phosphonolipids, where 2-aminoethyl phosphonate (AEPn) is analogous to ethanolamine phosphate as the constituent of phospholipids. In addition, Pn are constituents of polysaccharides, glycoproteins, glycolipids, and several antibiotics (25). Furthermore, large amounts of manmade Pn enter the environment (24). Pn utilization polypeptides in *Escherichia coli* are specified by the *phnCDEFGHIJKLMNOP* operon (15). Of the 14 cistrons, one (*phnF*) encodes a repressor protein, as inferred from sequence alignment (6); three (*phnCDE*) encode an ABC transport system for Pn (22); seven (*phnGHIJKLM*) have been postulated to encode the carbon-phosphorus (CP) lyase activity (26); and three (*phnNOP*) have been postulated to encode "auxiliary enzymes." Among the latter genes, the *phnO* gene has been shown to specify an enzyme with aminoalkylphosphonate *N*-acetyltransferase activity (5). We previously identified the product of the *phnN* gene as an enzyme capable of catalyzing the phosphorylation of ribose 1,5-bisphosphate to 5-phosphoribosyl α-D-1-diphosphate (ribose 1,5-bisphosphate phosphokinase; EC2.7.4.23) (11). The relation of these two compounds to Pn catabolism remains to be established. Since both the substrate and the product of ribose 1,5-bisphosphate phosphokinase are phosphorylated compounds, we inferred that at least some of the other intermediates of the pathway might be also phosphate esters. It might therefore be possible to identify these intermediates by labeling cells with radioactive phosphate ion in the presence of Pn followed by visualization by appropriate thin-layer chromatography (TLC) procedures. In addition, we employed mutants defective in individual genes of the pathway, and we looked for the accumulation of radiolabeled compounds as candidates for members of the *phn* pathway. Finally, the mutant strains used harbored the Δ*pstS605* allele to render the expression of the *phn* operon constitutive and, thus, independent of the phosphate supply.

Methods. The *E. coli* K-12 strains used and their construction are shown in Table 1. Cells were grown at 37°C in NZY broth (10), in phosphate-buffered AB minimal medium (64 mM P_i) (3), or in a low-phosphate, Tris-buffered medium, 03P (0.3 mM P_i) (12). Glucose (0.2%) was used as a carbon source; thiamine was added to give 1 mg/liter. Chloramphenicol was used at a concentration of 30 mg/liter. Cell growth was monitored as optical density (OD) at 436 nm in an Eppendorf PCP6121 spectrophotometer. Transduction by bacteriophage P1 (23) and transformation (14) were performed as previously described. A variant of the *phnK* gene specifying a polypeptide with a hexahistidine tag at the C-terminal end was amplified by using DNA of strain HO764 as the template, the four deoxyribonucleoside triphosphates, and Vent DNA polymerase (New England Biolabs, MA) in a thermocycler (model PC; Biometra, Göttingen, Germany). The oligodeoxyribonucleotides 5′-GGAAGGATCC*GAATTC*ATTAAAGAGGAGAAATTAA CT**ATG**AATCAACCGTTACTTTCGGTCAATAACCTGAC CC-3′ and 5′-TGGTTGGGATCCCGAG*CCATGG*TTATTA **ATGGTGATGGTGATGGTG**ATTCTGCAAAACCGATGAC ACCAGCAGCTGTGTATACGG-3′ (purchased at Eurofins Mwg Operon, Ebersberg, Germany) served as forward and reverse primers, respectively. Recognition sites for restriction endonucleases EcoRI and NcoI are shown in italics, and the initiation AUG-specifying sequence and hexahistidine tag-encoding sequence are shown in bold. The resulting DNA fragment was digested by EcoRI and NcoI and ligated to similarly digested DNA of pUHE23-2 (H. Bujard, personal communication). The resulting plasmid was designated pHO516. Nucleotide sequencing confirmed that pHO516 harbored the expected and correct sequence of the insert. Construction of the *phnP*-harboring plasmid pHO520 has previously been described (18). Cultures of strains HO2735 (Δ*phn*)/pHO516 (*phnK*$^+$) or HO2735 (Δ*phn*)/pHO520 (*phnP*$^+$) were grown exponentially at 37°C in NZY broth supplemented with ampicillin (100 mg/liter) and tetracycline (10 mg/liter). Isopropyl-1-thio-β-D-galactopyranoside was added to give 0.5 mM at an OD_{436} of 1.5 to induce expression of *phnP* or *phnK*. After 4 h of incubation at 37°C, cells were harvested by centrifugation, washed with 0.9% saline, concentrated 25-fold in 50 mM potassium phosphate buffer, pH 7.6, and broken in an ultrasonic disintegrator (Measuring and Scien-

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TABLE 1. Bacterial strains

Strain	Genotype[a]	Reference or construction
BW14894	F$^-$ Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30*	26
BW17485	F$^-$ Δ(*fumCA? manA uidA add*) *phnP54*::Tn*phoA′-1*[a]	15
BW17572	F$^-$ Δ(*fumCA? manA uidA add*) *phnO38*::Tn*phoA′-9*[a,b]	15
BW20274	F$^-$ Δ*uidA5 phnN45*::Tn*phoA′-3*[a,b]	11
BW26904	F$^-$ *lamB rph-1* Δ*pstS605*::*cat*	4
HO340	F$^-$ *tyrT*(As)[c]	17
HO764	F$^-$ *deoD gsk-3 udp*[c]	20
HO1088	F′ *tyrT*(As) *deoD gsk-3 udp prs-4*::Kan^{rc}/F *lacI*^{q} Tet^r	13
HO1168	F$^-$ *tyrT*(As) *deoD gsk-3 udp* Δ*prs-4*::Kan^r (*pst-phoU*)*1001 phoA528*::Tn*5*[c]	9
HO1182	F$^-$ *tyrT*(As) *deoD gsk-3 udp* (*pst-phoU*)*1001 phoA528*::Tn*5*[c]	P1(HO340) × HO1168; Prs$^+$
HO2531	F$^-$ Δ(*fumCA? manA uidA add*) *phnE15*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2532	F$^-$ Δ(*fumCA? manA uidA add*) *phnF23*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2533	F$^-$ Δ(*fumCA? manA uidA add*) *phnG35*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2534	F$^-$ Δ(*fumCA? manA uidA add*) *phnH13*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2535	F$^-$ Δ(*fumCA? manA uidA add*) *phnI40*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2536	F$^-$ Δ(*fumCA? manA uidA add*) *phnJ14*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2537	F$^-$ Δ(*lac*)X*74* Δ*phoA532 phnK6*::Tn*5-112* Δ*pstS605*::*cat*[b]	1
HO2538	F$^-$ Δ(*fumCA? manA uidA add*) *phnL39*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	1
HO2540	F$^-$ Δ*uidA5 phnN45*::Tn*phoA′-3* Δ*pstS605*::*cat*[a,b]	P1(BW26904) × BW20274; Cml^r
HO2541	F$^-$ Δ(*fumCA? manA uidA add*) *phnO38*::Tn*phoA′-9* Δ*pstS605*::*cat*[a,b]	P1(BW26904) × BW17572; Cml^r
HO2542	F$^-$ Δ(*fumCA? manA uidA add*) *phnP54*::Tn*phoA′-1* Δ*pstS605*::*cat*[a]	P1(BW26904) × BW17483; Cml^r
HO2545	F$^-$ Δ(*lac*)χ*74* Δ*phoA532* Δ(*mel-proP-phnCDEFGHIJKLMNOP*)*2*::Tn*5*seq1/132 Δ*pstS605*::*cat*	1
HO2568	F$^-$ Δ*uidA5 rph-1 rpoS396*(Am) Δ*pstS605*::*cat*[a]	1
HO2680	F$^-$ Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30* Δ*pstS605*::*cat*	P1(BW14894) × HO2545; Mel$^+$
HO2735	F′ Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30*/F *lacI*^{q} Tet^r	BW14894, conjugation (16) with HO1088; Tet^r Prs$^+$

[a] Also contains Δ(*lac*)χ*74* Δ*phoA532 phn-10*::*uidA2-aadA*.
[b] The *phn* lesion is nonpolar.
[c] Also contains *araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi*.

tific Equipment, Ltd., London, United Kingdom). Cell debris was removed by centrifugation.

^{32}P labeling of Pn degradation pathway intermediates was performed with cells growing exponentially in 03P medium. At an OD_{436} of 0.1, 0.74 MBq of $^{32}P_i$ (Nex 053; Perkin Elmer, Fremont, CA) was added to 2 ml of cell culture. Cell growth was followed in a parallel, unlabeled culture. After an additional two to three doublings, methyl phosphonate (MePn), AEPn, or formyl phosphonate (FoPn) (Sigma-Aldrich, Brøndby, Denmark) was added at a concentration of 2 mM. Samples were taken at an OD_{436} of 1.0 to 1.2; 200 μl culture was either extracted by the addition of 40 μl 2 M formic acid or centrifuged at 4°C for 2 min, followed by removal and storage of the supernatant fluid at −20°C. For TLC, cellulose-polyethyleneimine-coated plastic sheets, prepared as previously described (21), or commercial Baker-flex plates (J. T. Baker, Greisheim, Germany) were used. Radioactivity was quantified with the Cyclone Storage Phosphor System (PerkinElmer, Wellesley, MA).

Preparation of substrate for PhnP and assay of PhnP activity. The supernatant fluids of cultures of strain HO2542 (*phnP*) grown in the presence of $^{32}P_i$ and MePn or AEPn as described above were used as substrates after heating to 95°C for 5 min and centrifugation to remove debris. The activity of PhnP was assayed as follows. The supernatant fluids containing MePn- or AEPn-generated radiolabeled intermediates were mixed with 1 M potassium phosphate buffer, pH 7.6, and water to give a reaction cocktail with a potassium phosphate concentration of 62.5 mM. An 80-μl aliquot of this reaction cocktail (prewarmed at 37°C) was mixed with 20 μl of the PhnP (or PhnK)-containing extract (prewarmed at 37°C) and incubated at 37°C. Samples (10 μl) were mixed with 0.33% formic acid (5 μl) to stop the reaction. This 15 μl was applied to a polyethyleneimine TLC chromatography plate.

Accumulation of compounds in *phn* mutants. Preliminary analysis showed that several *phn* mutants accumulated one or more radiolabeled compounds when grown in the presence of Pn. We first analyzed mutants defective in the *phn* cistrons postulated to specify auxiliary enzymes, i.e., *phnN* (HO2540), *phnO* (HO2541), or *phnP* (HO2542) strains, for the accumulation of radiolabeled compounds in the presence of MePn, AEPn, or FoPn and included a strain defective in CP-lyase (the *phnH* strain HO2534) as well. In addition, a strain deleted for the *phn* operon (HO2680) and a strain harboring a wild-type *phn* operon (HO2568) were also analyzed. The majority of the compounds accumulated were found in the culture medium. Figure 1 shows the result of chromatography of the supernatant fluids of cultures of the six strains supplemented with any of the three Pn compounds (MePn, AEPn, or FoPn) or with no Pn. Clearly, the *phnP* strain (HO2542) (Fig. 1, lanes 21 to 24) accumulated two compounds when fed with MePn or AEPn. Two or more compounds accumulated in HO2542 when fed with FoPn. None of these compounds were present in the unsupplemented culture of HO2542 or in supplemented or unsupplemented cultures of strain HO2568 (*phn*$^+$ Δ*pstS*) (Fig. 1, lanes 1 to 4) or strain HO2680 (Δ*phn* Δ*pstS*) (Fig. 1, lanes 5 to 8). The spots of the relevant compounds are designated "S," "A," "B," "C," and "D." Compound S was found in all three Pn-supplemented cultures of strain HO2542. In contrast, strain HO2541 (*phnO* Δ*pstS*) (Fig. 1, lanes 17 to 20) did not accumulate any of these compounds. In fact, with this strain there was no difference in the chromatography pattern whether Pn

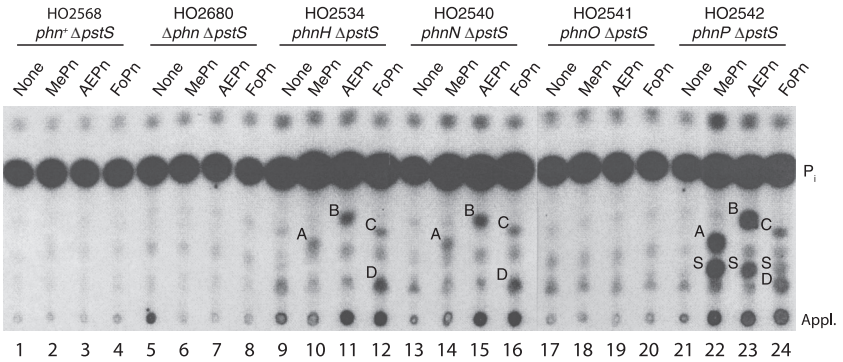

FIG. 1. Chromatography of supernatant fluids of cultures of *phn* Δ*pstS* strains grown with various Pn. The chromatogram was developed in methanol to the application line, followed by 1 M acetic acid (additional 2 cm) and 0.3 M lithium chloride-0.9 M acetic acid (additional 15 cm) (7, 21). A panel of four lanes represents each strain, indicated by designation and relevant genotype at the top. Each panel shows the chromatography of supernatant fluids of cultures grown in the absence of Pn or grown with MePn, AEPn or FoPn. "P_i" and "Appl." indicate the position of $^{32}P_i$ and the application line, respectively.

was added or not, and the results of chromatography resembled those for the *phn*$^+$ Δ*pstS* and Δ*phn* Δ*pstS* strains. The apparent tiny differences in the intensities of the various spots in the labeling pattern of strain HO2541 versus those of strains HO2568 and HO2680 were caused by small fluctuations in pipetting the radioactive material. The *phnN* Δ*pstS* and *phnH* Δ*pstS* strains (HO2540 and HO2534, respectively) (Fig. 1, lanes 9 to 12 and 13 to 16, respectively) revealed identical labeling patterns, and both strains accumulated the "uppermost" compound found also with the *phnP* Δ*pstS* strain (HO2542), i.e., the compounds represented by the spots designated "A," "B," and "C" as well as the "D" spot, whereas spot "S" was absent. The fact that the spots designated "S" represented the same compound was confirmed by two-dimensional chromatography, by which the compounds were cochromatographed (data not shown). The amount of each compound formed by strain HO2542 was determined (Table 2). Essentially all of compound S was found in the supernatant fluid in the cultures grown with MePn or AEPn. The presence of the various compounds in the culture fluid is consistent with the finding of ethylphosphonate α-D-ribofuranose in the culture fluid of cells grown with ethylphosphonate (2) and with the finding that methane production from MePn occurs in the absence of the Pn ABC transport system and, thus, that at least part of the CP-lyase pathway occurs in the periplasm (26).

It has been suggested that the CP-lyase pathway involves the attachment of Pn compounds to a small molecule, a cofactor, very likely a ribose derivative, before CP-bond cleavage (11). Compounds A, B, and D may represent such intermediates of the pathway. According to that suggestion, compound A may contain a methylphosphonyl moiety attached to the cofactor, whereas compound B may contain an aminoethylphosphonyl moiety attached to the cofactor. Compound B therefore may contain an amino group, which would be protonated under the acidic cromatographic conditions, resulting in a larger retention factor (R_f) than that of compound A with the anion exchanger used. Similarly, compound D may contain a formylphosphonyl moiety attached to the cofactor, which would introduce an additional negative charge, resulting in a lower R_f value. Compound C does not fit in this scheme and may represent a compound accumulating for other reasons.

Chromatographic identification of the substrate and product of PhnP. Since compound S appeared with all three Pn compounds in the *phnP* strain (HO2542), we inferred that this compound could be the substrate for the *phnP* gene product. Indeed, compound S present in the supernatant fluid of MePn-grown cells of strain HO2542 disappeared upon addition of a cell extract containing PhnP. Simultaneously, a new radiolabeled compound, designated "P," with a lower R_f value appeared in the chromatogram (Fig. 2a). In contrast, a cell extract containing PhnK was unable to convert compound S. We therefore concluded that compound S is the substrate for PhnP and that compound P is the product of PhnP. Applying a dilution of the extract containing PhnP, we showed that the

TABLE 2. Pool sizes of compounds accumulating in strain HO2542 (*phnP*) after addition of Pn[a]

Pn	Compound[b]	Pool size (μmol/[liters × OD_{436}]) in fraction		
		Whole culture[c]	Supernatant fluid	Cells[d]
MePn	S	16	15	1
	A	14	11	3
AEPn	S	8.8	8.9	0
	B	34	21	13
FoPn	S	1.9	1.4	0.5
	C	3.2	2.3	1.1
	D	1.9	2.0	0

[a] Cells were grown in 03P medium supplemented with glucose and the indicated Pn, labeled with $^{32}P_i$, and extracted as described in the text. Pool sizes were determined by two-dimensional TLC and were calculated with the assumption that a single phosphorus atom originated from P_i. The first dimension consisted of methanol, 1 M acetic acid, 0.3 M lithium chloride-0.9 M acetic acid (system described in the legend to Fig. 1). After being dried and trimmed, the chromatogram was neutralized in methanol containing Tris base (1.2 g/liter) (500 ml for each of two 20- by 20-cm plates), washed in methanol, dried, and submitted to chromatography (18 cm) in the second dimension in a solvent consisting of 6 g of sodium borate (decahydrate), 3 g of boric acid, and 25 ml of ethylene glycol in 70 ml of water (8, 21).

[b] The compounds are designated as in Fig. 1.

[c] The culture was extracted directly with formic acid.

[d] The content of a compound in the cells was calculated as the difference between the content in whole culture and that in the supernatant fluid.

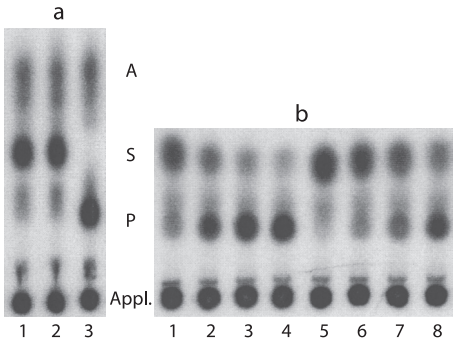

FIG. 2. Chromatographic identification of the substrate and product of the PhnP enzyme. (a) A reaction cocktail consisting of the supernatant fluid of strain HO2542 (*phnP* Δ*pstS*) grown in the presence of $^{32}P_i$ and MePn together with buffer was prepared as described in the text. To analyze the conversion of the compound labeled "S," this reaction cocktail was treated as follows: for lane 1, the reaction cocktail was mixed with 50 mM potassium buffer, pH 7.6, followed by application to the chromatography plate; for lane 2, the reaction cocktail was mixed with an extract of strain HO2735/pHO516 (*phnK*$^+$) and incubated at 37°C for 120 min, followed by application to the chromatography plate; and for lane 3, the reaction cocktail was mixed with an extract of strain HO2735/pHO520 (*phnP*$^+$) and incubated at 37°C for 1 min, followed by application to the chromatography plate. (b) The reaction cocktail was incubated with an extract of strain HO2735/pHO516 (*phnP*$^+$) at 37°C as follows: for lanes 1 to 4, a 10-fold-diluted extract was used; and for lanes 5 to 8, a 25-fold-diluted extract was used. Samples were removed for chromatography after incubation for 1 min (lanes 1 and 5), 3 min (lanes 2 and 6), 6 min (lanes 3 and 7), or 15 min (lanes 4 and 8). Compounds S and A are designated as in Fig. 1. The TLC system is identical to that used for Fig. 1 except that chromatography was extended to improve the separation of compounds with low R_f values: a piece of 3MM paper (Whatman, Maidstone, United Kingdom) was cut to the size of the TLC plate, folded three times and thoroughly applied to the top of the plate. Chromatography was terminated when the attached paper had been fully wetted.

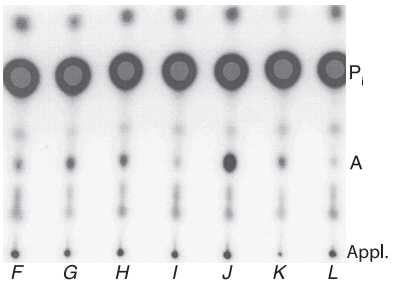

FIG. 3. Accumulation of labeled compounds by various *phn* Δ*pstS* strains grown in the presence of MePn. Cells were grown and the supernatant fluids were prepared as described in the text. The chromatography system was the same as that used for the analysis shown in Fig. 1. The strains used were HO2532 (*phnF* Δ*pstS*), HO2533 (*phnG* Δ*pstS*), HO2534 (*phnH* Δ*pstS*), HO2535 (*phnI* Δ*pstS*), HO2536 (*phnJ* Δ*pstS*), HO2537 (*phnK* Δ*pstS*), and HO2538 (*phnL* Δ*pstS*). The *phn* lesion of each strain is indicated by letters in italics below each lane. The spots are designated as in Fig. 1 and 2.

rate of conversion of compound S to compound P was dependent on enzyme concentration and time (Fig. 2b). From the data obtained with the 25-fold-diluted enzyme represented in Fig. 2b, we calculated the activity responsible for the disappearance of compound S under these assay conditions as 6.6 nmol/(min × ml of extract) and the activity responsible for the appearance of compound P as 7.3 nmol/(min × ml of extract), assuming that the compounds contained a single radiolabeled phosphorus atom. Thus, the two compounds disappeared and appeared in stoichiometric amounts. Compound A was left untouched by the cell extracts. Similar results were obtained with the supernatant fluid of strain HO2542 (*phnP* Δ*pstS*) grown in the presence of $^{32}P_i$ and AEPn, i.e., the PhnP containing extract removed compound S, and a compound with an R_f value identical to that of compound P appeared, whereas compound B was left untouched, and as before, the PhnK-containing extract did not convert either compound S or compound B (data not shown).

The fact that the conversion of compound S to compound P occurs in the absence of any added cofactor or cosubstrate indicates that the reaction is a rearrangement of the substrate, for example, a hydrolysis. Furthermore, polyethyleneimine is an anion exchanger, and thus, separation occurs primarily on the basis of charge. Consequently, compound P has at least one more negative charge than compound S. This observation is consistent with the fact that *phnP* specifies an enzyme with phosphodiesterase activity. The enzyme is capable of hydrolyzing 2′,3′-cyclic nucleotides, like 2′,3′-cyclic CMP, 2′,3′-cyclic AMP, 2′,3′-cyclic GMP, and, to a much lesser extent, 3′,5′-cyclic AMP and 3′,5′-cyclic GMP (19). Compound S, the true or physiologic substrate for PhnP, is different from either of these cyclic nucleotides. Thus, in the chromatographic system containing lithium chloride in acetic acid, the R_f value of compound S was 0.11, whereas that of 2′,3′-cyclic CMP was appreciably larger, 0.61. The two other compounds, 2′,3′-cyclic AMP and 2′,3′-cyclic GMP, were not submitted to chromatography, but the R_f values of 5′-AMP and 5′-GMP were 0.31 and 0.41, respectively, and the R_f values of the corresponding cyclic nucleotides would be even larger due to the reduced charge of the cyclic nucleotides. Likewise, the R_f value of 3′,5′-cyclic AMP is 0.45.

In addition to the extract of the strain that specifically overexpressed the *phnP* gene, we found that an extract of the Pho regulon-constitutive strain HO1182 (*phn*$^+$ *pst-phoU*) was also able to convert compound S to compound P. Similarly, extracts of *pst-phoU* strains containing in addition a *phnF* (strain HO2532), *phnG* (HO2533), *phnH* (HO2534), *phnI* (HO2535), *phnJ* (HO2536), *phnK* (HO2537), or *phnL* (HO2538) knockout allele were able to convert compound S to compound P. In contrast, neither extracts of a Δ*phn pst-phoU* (HO2680) nor extracts of a *phnP pst-phoU* strain (HO2542) were able to convert compound S to compound P. Altogether, compound S is specific for PhnP, and no other *phn* specified gene product was able to convert the compound.

Accumulation of intermediates in other *phn* mutants grown with $^{32}P_i$ and MePn. The *pst-phoU* strains containing knockout alleles of *phnF* (HO2532), *phnG* (HO2533), *phnH* (HO2534), *phnI* (HO2535), *phnJ* (HO2536), *phnK* (HO2537), or *phnL* (HO2538) were analyzed for the accumulation of radiolabeled MePn degradation intermediates. The results revealed that each of the mutants accumulated compound A, although in various amounts (Fig. 3). The concentrations of compound A in the culture media of the various strains were as follows: for *phnF*, 0.9; for *phnG*, 3.4; for *phnH*, 3.2; for *phnI*, 0.8; for *phnJ*, 11; for *phnK*, 2.0; and for *phnL*, 0.5 μmol/(liters × OD_{436}).

Apart from the variations in size of compound A, the chromatographic patterns of the culture media of the *phnF*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, and *phnL* strains are similar to that of the *phnH* strain represented in Fig. 1. Compound A did not accumulate in the absence of MePn (data not shown). In addition, each supernatant fluid was incubated with a cell extract of strain HO1182 (*pst-phoU*) to see if accumulated compounds could be converted. Conversion both in the presence and in the absence of ATP was analyzed. There was no change in the chromatography pattern after this incubation. Analysis of the accumulation of intermediates in a *phnM* strain revealed that compound A did not accumulate. However, this result is inconclusive because the available *phnM28* allele is polar and results in a $PhnM^{-}N^{-}O^{-}P^{-}$ phenotype (15) (data not shown).

Conclusions. In situ radiolabeling of *E. coli* metabolic intermediates and cell signaling molecules with ^{32}P phosphate ion, combined with knockout of individual CP-lyase genes and a simple TLC method, has identified a number of potential phosphorylated compounds related to organophosphonate metabolism, including the in vivo substrate for PhnP. Along with previous studies describing a substrate for PhnN (11), and the accumulation of ethylphosphonate α-D-ribofuranose in *E. coli* subsisting on ethylphosphonate (2), the present work strengthens the case that organophosphonates are processed by CP-lyase through a number of metabolic intermediates, prior to and following the cleavage of the CP bond. Isolation and full characterization of these compounds will be necessary to reconstruct the CP-lyase pathway, identify substrates for individual CP-lyase enzymes, and ultimately probe the mechanism of the remarkable CP bond cleavage step.

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