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Physiological Role of *phnP*-specified Phosphoribosyl Cyclic Phosphodiesterase in Catabolism of Organophosphonic Acids by the Carbon−Phosphorus Lyase Pathway

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Supporting Information

ABSTRACT: In *Escherichia coli*, internalization and catabolism of organophosphonic acids are governed by the 14-cistron *phnCDEFGHIJKLMNOP* operon. The *phnP* gene product was previously shown to encode a phosphodiesterase with unusual specificity toward ribonucleoside 2′,3′-cyclic phosphates. Furthermore, *phnP* displays shared synteny with *phnN* across bacterial *phn* operons. Here the role of PhnP was examined by ^{31}P NMR spectrometry on the culture supernatants of *E. coli phn* mutants grown in the presence of alkylphosphonic acid or phosphite. The addition of any of these alkylphosphonic acids or phosphite resulted in the accumulation of α-D-ribosyl 1,2-cyclic phosphate and α-D-ribosyl 1-alkylphosphonate in a *phnP* mutant strain. Additionally, α-D-ribosyl 1-ethylphosphonate was observed to accumulate in a *phnJ* mutant strain when it was fed ethylphosphonic acid. Purified PhnP was shown to regiospecifically convert α-D-ribosyl 1,2-cyclic phosphate to α-D-ribosyl 1-phosphate. Radiolabeling studies revealed that 5-phospho-α-D-ribosyl 1,2-cyclic phosphate also accumulates in a *phnP* mutant. This compound was synthesized and shown to be regiospecifically converted by PhnP to α-D-ribosyl 1,5-bisphosphate. It is also shown that organophosphonate catabolism is dependent on the synthesis of 5-phospho-α-D-ribosyl 1-diphosphate, suggesting that this phosphoribosyl donor is used to initiate the carbon−phosphorus (CP) lyase pathway. The results show that 5-phospho-α-D-ribosyl 1,2-cyclic phosphate is an intermediate of organophosphonic acid catabolism, and it is proposed that this compound derives from C−P bond cleavage of 5-phospho-α-D-ribosyl 1-alkylphosphonates by CP lyase.

INTRODUCTION

The catabolism of organophosphonic acids by the carbon−phosphorus (CP) lyase pathway of *Escherichia coli* and numerous other bacteria requires the gene products of the 14-cistron *phnCDEFGHIJKLMNOP* operon.[1] The genetics of phosphonic acid utilization in *E. coli* has been described in great detail.[2,3] Internalization of organophosphonic acid requires the *phnCDE* genes, which specify an ATP binding cassette transporter with the *phnD* gene encoding the periplasmic binding protein for organophosphonic acid.[4,5] The *phnF* gene appears to encode a repressor of *phn* operon expression as evaluated by amino acid sequence similarity to the *phnF* gene product of *Mycobacterium smegmatis*,[6] whereas *phnO* encodes an enzyme with N-acetyltransferase activity toward aminoalkylphosphonic acids.[7] The genes *phnGHIJKLM* have been postulated to encode C−P bond-cleaving activity by CP lyase.[1,3,4,8] Thus, lack of any of these seven cistrons results in lack of production of methane from methylphosphonic acid.[8,9] Cell growth is also differentially affected by mutations. *E. coli* strains with transposon insertions in *phnF*, *phnN*, or *phnO* are phosphonic acid growth-proficient, whereas strains with transposon insertions in *phnC*, *phnD*, *phnE*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM*, or *phnP* are phosphonic acid growth-deficient.[3] Furthermore, feeding *E. coli* with ethylphosphonic acid (**1b**) leads to the accumulation of α-D-ribosyl 1-ethylphosphonate (**2b**, Scheme 1).[10] The *phnN* gene specifies α-D-ribosyl 1,5-bisphosphate phosphokinase, which produces 5-phospho-α-D-ribosyl 1-diphosphate (PRPP, **4**) (Scheme 2), a phosphoribosyl donor in nucleotide, histidine, and tryptophan biosynthesis.[11] As all of these compounds are ribose derivatives, organophosphonic acid catabolism by CP lyase likely involves a pathway with ribosylation or phosphoribosylation of the organophosphonic acid prior to C−P bond cleavage. Finally, the *phnP* gene has been shown to encode a phosphodiesterase of the β-lactamase superfamily with specificity toward ribonucleoside 2′,3′-cyclic phosphates.[12]

In the present work we show that 5-phosphoribosyl 1,2-cyclic phosphate (**5b**) is an intermediate in the organophosphonic acid degradation pathway, and that this compound, as well as its dephosphorylated analogue, α-D-ribosyl 1,2-cyclic phosphate, is a substrate for the phosphodiesterase PhnP. This finding provides a link of the substrate of the PhnP-catalyzed reaction to 5-phospho-α-D-ribosyl 1-alkylphosphonates, which we propose are the intermediates that undergo C−P bond cleavage by CP lyase.

RESULTS

Accumulation of Phosphorus-Containing Compounds in *phn* Mutants of *E. coli*. Mutants of *E. coli* with transposon

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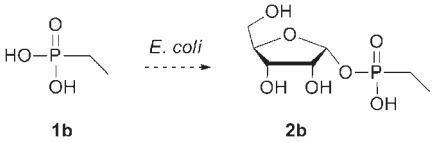
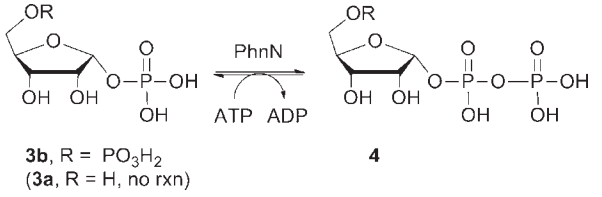
Scheme 1. Incorporation of Ethylphosphonic Acid into Ribose by *E. coli*



Scheme 2. Reaction of *phnN*-Specified α-D-Ribosyl 1,5-Bisphosphate Phosphokinase



insertions in *phnE* (strain HO2531), *phnG* (HO2533), *phnH* (HO2534), *phnI* (HO2535), *phnJ* (HO2536), *phnK* (HO2537), *phnL* (HO2538), *phnM* (HO2539), *phnN* (HO2540), *phnO* (HO2541), or *phnP* (HO2542), as well as a Δ*phn* strain (HO2680) and a *phn*$^+$ strain (HO2568), were used to probe organophosphonate catabolism. All strains also contain a Δ*pstS* allele to render *phn* operon expression constitutive and independent of the phosphate supply.[13,14] The strains were grown in the presence of methylphosphonic acid (**1a**). P_i was also added as the mutant strains were unable to utilize **1a** as a P_i source. The used growth medium was subsequently analyzed for phosphorus compounds by ^{31}P NMR spectroscopy. The *phn*$^+$, *phnE*, *phnG*, *phnH*, *phnI*, *phnK*, *phnL*, *phnM*, *phnN*, *phnO*, and Δ*phn* mutants did not exhibit ^{31}P NMR signals other than **1a** (δ 23−24 ppm), indicating the absence of phosphorus-containing intermediates, at least not in quantities allowing detection by NMR. This is not surprising for the *phn*$^+$ strain, which constitutively expresses an intact and proficient CP lyase pathway. Likewise, the *phnO* gene is important only for the catabolism of aminoalkylphosphonates, and *phnN* was shown previously to be dispensable for cell growth on alkylphosphonates. However, a *phnJ* mutant revealed a second peak at δ 26 ppm in addition to residual **1a**. In contrast, the *phnP* strain produced the δ 26 ppm peak as well as a third peak at δ 18.6 ppm (Figure 1a). All mutants, with the exception of *phnP*, exhibited residual P_i at δ 1.2−1.8 ppm, arising from the P_i that was included in the culture medium to enable bacterial growth. Similar results were obtained with ethylphosphonic acid (**1b**) (data not shown).

To further analyze the conversion of phosphonic acids, strain HO2542 (*phnP*) was grown in the presence of various organophosphonic acids or phosphite, and analyses similar to those described above were conducted (Table 1). All added compounds consistently produced a peak at δ 18.6 ppm. As well, with the exception of phenylphosphonic acid (**1f**), all added compounds produced a unique peak downfield from the residual phosphonic acid. As explained below, these are likely α-D-ribosyl 1-phosphonic acid derivatives of the generic structure **2**. For 2-aminoethylphosphonic acid (**1e**) two downfield peaks appeared (δ 23.6 and 19.4 ppm), one of which may represent an N-acetyl derivative as a result of the activity of the *phnO* gene

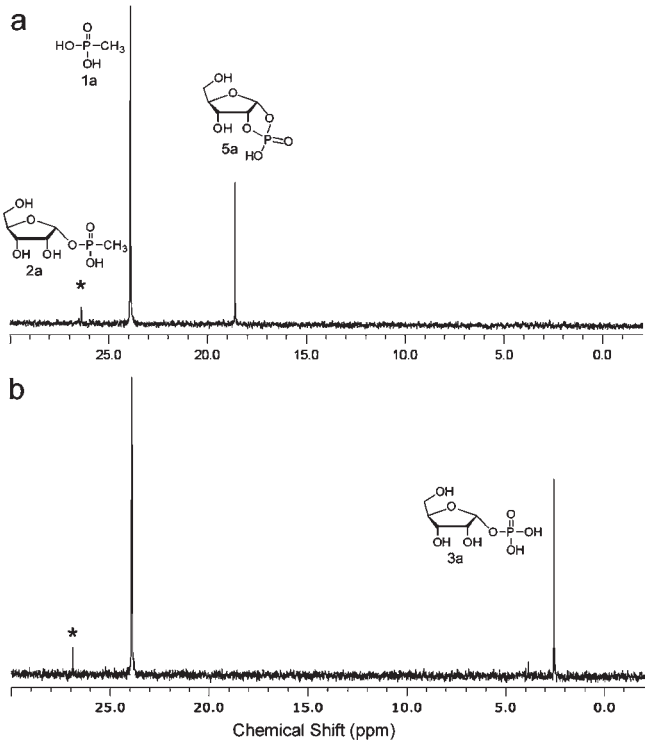


Figure 1. ^{31}P NMR spectra of the culture supernatant of HO2542 (*phnP*) grown in the presence of methylphosphonic acid, (a) before and (b) after incubation with purified PhnP. The peak for **2a** is indicated with an asterisk (*).

product.[7] The levels of the putative α-D-ribosyl 1-phosphonic acid derivatives, estimated by comparison to a known amount of P_i in the sample, were quite low. However, the compound represented by the δ 18.6 ppm peak constituted up to approximately 20% of the remaining phosphonic acid. In some cases a small peak at δ 1.2−2 ppm, representing unused P_i remaining in the growth medium, was observed and is not included in Table 1.

Analysis of Intermediates Accumulating in *phn* Mutant Strains. The fact that a chemical shift of δ 18.6 ppm was observed in the culture supernatant of only the *phnP* strain, and that this peak was observed with a variety of alkylphosphonic acids and phosphite, strongly indicates that this compound is a common intermediate of the phosphonic acid degradation pathway *after* the cleavage of the C−P bond (or H−P bond in the case of phosphite). In contrast, the fact that the chemical shifts of the low-field peaks varied according to the added phosphonic acid strongly indicates that these compounds are phosphonic acid derivatives and therefore intermediates of the phosphonic acid degradation pathway *before* cleavage of the C−P bond. The ^{31}P NMR chemical shift of δ 18.6 ppm suggested that this compound was α-D-ribosyl 1,2-cyclic phosphate (**5a**) (Table 1) due to its similarity to the value reported for 5-phosphoribosyl 1,2-cyclic phosphate (**5b**) (Scheme 3).[15,16] The absence of a peak at ∼4 ppm indicates that **5a** lacks a 5-phosphate ester. The coupling constants $J_{P1,2-H1}$ and $J_{P1,2-H2}$ measured for **5a**, 17 and 4.3 Hz, respectively, also compare well with literature values.[15] We synthesized **5b** by reaction of **4** with barium acetate under alkaline conditions (Scheme 3), and likewise found essentially identical chemical shifts and coupling constants for the anomeric phosphate when compared to **5a** (Table 2).

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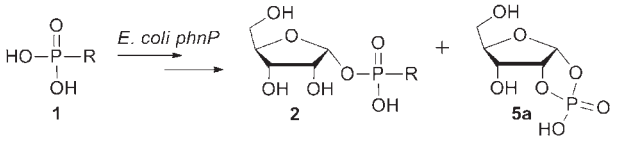
Table 1. ^{31}P NMR Chemical Shifts in Supernatant Fluids of Strain HO2542 (*phnP*) Grown in the Presence of Various Organophosphonic Acids or Phosphite



Addition	chemical shift (ppm)	concn (mM)[a]	assignment
R = CH_3, **1a**	26.4	0.02	**2a**, R = CH_3
	23.9	1.62	**1a**
	18.6	0.36	**5a**
R = CH_3CH_2, **1b**	30.3	0.11 (0.08)[b]	**2b**, R = CH_3CH_2
	27.6	1.83 (0.22)	**1b**
	18.6	0.06 (<0.01)	**5a**
R = $CH_3CH_2CH_2$, **1c**	28.7	0.10	**2c**, R = $CH_3CH_2CH_2$
	25.8	1.90	**1c**
	18.6	<0.01	**5a**
R = H_2NCH_2, **1d**	18.6	0.08	**5a**
	13.6	0.24	**2d**, R = H_2NCH_2 or $AcNHCH_2$
	9.2	1.68	**1d**
R = $H_2NCH_2CH_2$, **1e**	23.6	0.02	**2e**, R = $H_2NCH_2CH_2$ or $AcNHCH_2CH_2$
	19.4	0.36	**2f**, R = $H_2NCH_2CH_2$ or $AcNHCH_2CH_2$
	18.5	<0.01	**5a**
	16.8	1.62	**1e**
R = Ph, **1f**	18.6	0.02	**5a**
	11.9	1.98	**1f**
R = H, **1g**	18.6	0.07	**5a**
	13.3	0.03	**2g**, R = H
	3.0	1.90	**1g**

[a] Concentrations estimated by integration relative to a known concentration of P_i. [b] Data in parentheses were obtained with cells grown in the presence of 0.3 mM ethylphosphonic acid.

The structure of the compound represented by the δ 26 ppm peak resulting from HO2542 (*phnP*) grown on **1a** was not directly determined (Figure 1a). However, the analogous compound (δ 30.3 ppm, Table 1) produced by ethylphosphonic acid (**1b**) was purified and characterized. Interestingly, strain HO2542 (*phnP*) grown in the presence of 0.3 mM **1b** resulted in relatively higher production of the δ 30.3 ppm compound, as well as a relatively lower production of **5a**, compared to that produced in the presence of 2 mM **1b**. The δ 30.3 ppm compound was purified by ion-exchange chromatography and shown by ^{1}H and ^{31}P NMR spectroscopy to agree with the data reported previously for **2b** (see Experimental Procedures).[10] By analogy, the low-field peaks observed in supernatant fluids of strain HO2542 (*phnP*) grown in the presence of methylphosphonic acid (**1a**), propylphosphonic acid (**1c**), aminomethylphosphonic acid (**1d**), or phosphite (**1g**) likely correspond to α-D-ribosyl 1-methylphosphonate (**2a**, δ 26.4 ppm), α-D-ribosyl 1-propylphosphonate (**2c**, δ 28.7 ppm), α-D-ribosyl 1-aminomethylphosphonate (**2d**, δ 13.6 ppm), or α-D-ribosyl 1-phosphite (**2g**, δ 13.3 ppm), respectively. Similarly, one of the low-field peaks observed after growth of strain HO2542 (*phnP*) with 2-aminoethylphosphonic acid (**1e**) likely corresponds to α-D-ribosyl 1-(2′-aminoethylphosphonate) or its N-acetylated derivative (**2e** and **2f**, δ 23.6 and 19.4 ppm, respectively).

α-D-Ribofuranosyl 1,2-Cyclic Phosphate and 5-Phospho-α-D-Ribofuranosyl 1,2-Cyclic Phosphate as Substrates for PhnP. Because PhnP was previously shown to have activity toward ribonucleoside 2′,3′-cyclic phosphates, we considered whether **5a** would also be a substrate for the enzyme. Addition of purified PhnP to the culture supernatant of HO2542 (*phnP*) grown on methylphosphonic acid (**1a**) resulted in the disappearance of the δ 18.6 ppm peak corresponding to **5a**, and the appearance of a peak at δ 2.5 ppm (Figure 1b). This new peak was shown to be α-D-ribosyl 1-phosphate (**3a**) by spiking the NMR sample with authentic material (data not shown), as well as by the observation of essentially identical J_{P1-H1} coupling constants with the authentic sample (Table 2).

All of the intermediates observed in *E. coli* culture supernatants appear to lack a C5 phosphate ester, as evidenced by the lack of a high-field peak at approximately 4 ppm (Figure 1a). In contrast, the *phnN*-specified α-D-ribosyl 1,5-bisphosphate phosphokinase is specific for a 5-phosphorylated substrate (Scheme 2).[11] We then considered the possibility that the compounds found in the culture supernatants arose by excretion from the cells with concomitant hydrolysis of the C5 phosphate ester. Therefore, **5b** was synthesized as shown in Scheme 3. Upon incubation of **5b** with purified PhnP, the δ 18.6 ppm peak representing the 1,2-cyclic phosphate disappeared, followed by the appearance of a peak of identical integral at δ 2.3 ppm. Analysis of **5b** and the reaction product by ^{1}H/^{31}P HMBC revealed regiospecific conversion of **5b** to α-D-ribosyl 1,5-bisphosphate (**3b**) (Scheme 3, spectra available as Supporting Information). The structure of **3b** is further confirmed by a single

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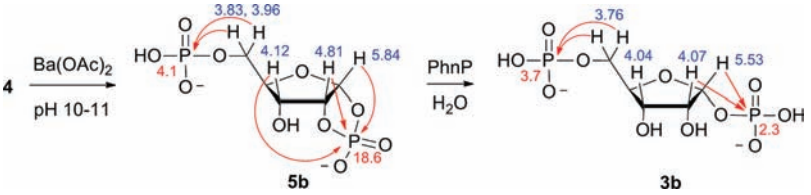
Table 2. ^{31}P NMR Chemical Shifts and $^{31}P-^{1}H$ Coupling Constants for Relevant Compounds[a]

compd	chemical shift (δ, ppm)			coupling constant (J, Hz)				
	1-P	1,2-P	5-P	$J_{P1,2-H1}$	$J_{P1,2-H2}$	J_{P1-H1}	J_{P5-H5a}	J_{P5-H5b}
α-D-ribosyl 1,2-cyclic phosphate **5a**[b]		18.6		17	4.5			
α-D-ribosyl 1-phosphate **3a**[c]	1.9					6.4		
α-D-ribosyl 1-phosphate **3a**[d]	2.1					6.5		
5-phospho-α-D-ribosyl 1,2-cyclic phosphate **5b**		18.7	4.1	17	4.3		5.0	5.0
α-D-ribosyl 1,5-bisphosphate **3b**[e]	2.3		3.7			6.2	4.7	4.7

[a] Chemical shifts were determined at pH 7.6. [b] Supernatant fluid of methylphosphonic acid (**1a**)-grown cells of strain HO2542 (*phnP*). [c] Product of incubation of α-D-ribosyl 1,2-cyclic phosphate **5a** with PhnP, Figure 1B. [d] Data obtained with in vitro synthesized, authentic α-D-ribosyl 1-phosphate (**3a**). [e] Product of incubation of **5b** with PhnP.

Scheme 3. In Vitro Synthesis of 5b Followed by Conversion by PhnP to 3b[a]



[a] Observed $^{1}H/^{31}P$ HMBC correlations are shown as red arrows.

downfield ^{1}H NMR signal for H1 (δ = 5.53 ppm, J_{H1-H2} = 4.1 Hz), which is consistent with the α-D-ribosyl phosphate configuration, whereas signals corresponding to a α/β mixture would be anticipated for the 2-phosphate ester. Therefore, the spectroscopic data are consistent with the regiospecific conversion of **5b** to **3b**.

5-Phospho-α-D-ribofuranosyl 1,2-Cyclic Phosphate (5b) as an Intermediate in Organophosphonic Acid Catabolism. To ascertain that **5b** is an intermediate of the CP lyase pathway, **5b** was synthesized in vitro in radiolabeled form (Scheme 3), whereas the physiological substrate of PhnP was isolated from a culture of strain HO2542 (*phnP*) as described under Experimental Procedures. Results of one-dimensional thin-layer chromatography (TLC) analysis of these two preparations are shown in Figure 2. In lanes 1 and 2 the radiolabeled compounds of the supernatant fluid of strain HO2542 (*phnP*) and the synthesized cyclic compound, respectively, were chromatographed, whereas in lane 3 the two preparations were mixed before chromatography. Although the chromatography of the in vitro synthesized compound is somewhat distorted (lane 2), possibly due a different sample pH, it is obvious from lane 3 that the in vitro synthesized compound, which is 5-phospho-α-D-ribosyl 1,2-cyclic phosphate (**5b**), and the in vivo produced physiological substrate of PhnP cochromatographed. We therefore conclude that the physiological substrate of PhnP and **5b** are the same compound and that **5b**, therefore, is an intermediate in organophosphonic acid catabolism.

Lack of Utilization of Phosphonic Acid by a Δ*prs* Strain. We analyzed the effect of a Δ*prs* allele on utilization of phosphonic acid as phosphate source. *E. coli* Δ*prs* strains are defective in PRPP synthase. Because PRPP (**4**) is required for biosynthesis of purine, pyrimidine, and pyridine nucleotides as well as the amino acids histidine and tryptophan, a Δ*prs* strain requires guanosine, uridine, histidine, tryptophan, and nicotinamide adenine dinucleotide (NAD).[17,18] Introduction of a

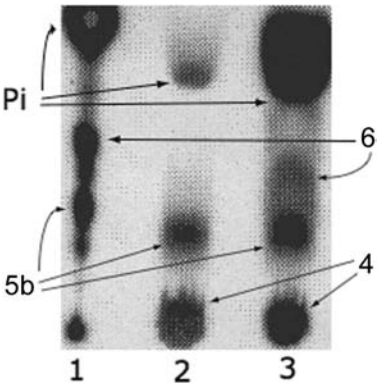


Figure 2. TLC on poly(ethylenimine)−cellulose of ^{32}P-labeled organophosphonic acid metabolites. Lane 1, supernatant fluid of strain HO2542 (*phnP*) grown in the presence of $^{32}P_i$ and unlabeled methylphosphonic acid; lane 2, **5b** formed after incubation of [^{32}P]PRPP (**4**) as described under Experimental Procedures; lane 3, mixture of equal amounts of the solutions applied to lanes 1 and 2. The identity of **6** is presumed (see text).

mutation of the *pstSCAB-phoU* operon into a Δ*prs* strain suppresses the NAD requirement due to constitutive expression of the *phnN* gene encoding the PRPP-producing enzyme α-D-ribosyl 1,5-bisphosphate phosphokinase (Scheme 2). In contrast, the requirement for guanosine, uridine, histidine, and tryptophan is retained in a Δ*prs pstSCAB-phoU* strain.[11,19] As described above, α-D-ribosyl 1,5-bisphosphate (**3b**) is the product of PhnP-catalyzed hydrolysis of **5b**, and, therefore **3b**, like **5b**, is an intermediate of phosphonic acid catabolism. We therefore hypothesized that the addition of phosphonic acid to cultures of *E. coli* might result in increased synthesis of **3b** and, subsequently, in increased synthesis of PRPP (**4**), resulting in alleviation of some or all of the nutritional requirements of a Δ*prs* strain. To test this hypothesis, strain HO3342 (Δ*prs* Δ*pstS*) was streaked

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on solid glucose Mops minimal medium containing methylphosphonic acid (**1a**) and P*i* together with guanosine, uridine, histidine, and tryptophan as well as on solid media lacking individually one of these latter four additions. However, there was no growth in the absence of guanosine, uridine, histidine, or tryptophan, whereas normal growth was observed in the presence of these four additions. Thus, contrary to our expectations, the addition of **1a** to strain HO3342 (Δ*prs* Δ*pstS*) did not alleviate the requirement for any of the compounds guanosine, uridine, histidine, or tryptophan, and thus, the presence of **1a** did not stimulate the synthesis of **4** by *phnN*-specified α-D-ribosyl 1,5-bisphosphate phosphokinase. Furthermore, after streaking and incubating strain HO3342 (Δ*prs* Δ*pstS*) on solid medium containing **1a** as sole P*i* source and with guanosine, uridine, histidine, and tryptophan present, no growth at all was observed, whereas normal growth was observed in the presence of P_i. In contrast, strain HO2568 (*prs*$^+$ Δ*pstS*) grew well in the presence of **1a** as phosphate source, and thus HO2568 utilized **1a** as a phosphate source. The lack of growth of strain HO3342 (Δ*prs* Δ*pstS*) with **1a** as phosphate source, therefore, is attributed to the lack of *prs*-specified PRPP synthase and, very likely, lack of production of **4**. This result shows that organophosphonic acid utilization is dependent on the synthesis of **4**, rather than **4** being a dedicated product of this catabolism.

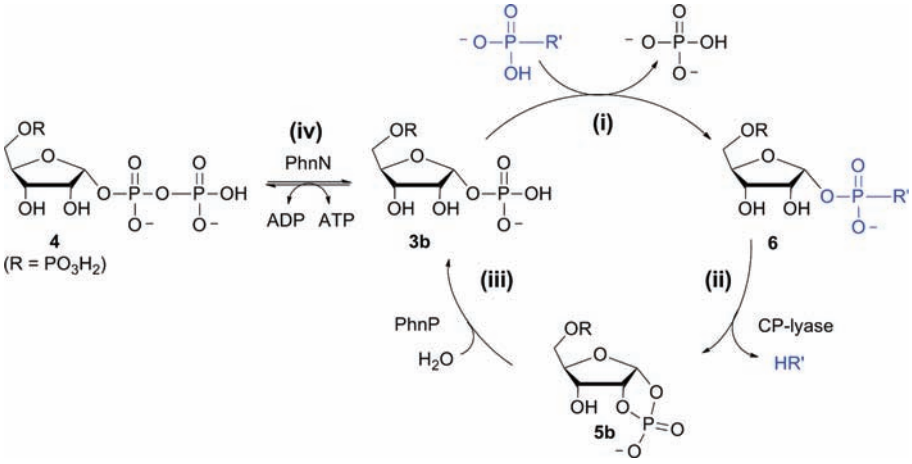
DISCUSSION

The *phnP* gene of *E. coli* specifies a metal ion-dependent phosphodiesterase of the *β*-lactamase superfamily.[12] The enzyme is active against the generic phosphodiesterase substrate bis(*p*-nitrophenyl) phosphate as well as ribonucleoside 2′,3′-cyclic phosphates, the latter hydrolyzed to the corresponding ribonucleoside 3′-phosphates. Apart from cyclic adenosine monophosphate (cAMP) and cyclic diguanylate, cyclic nucleotides are not prominent members of the cellular metabolism of *E. coli*. However, guanosine 5′-diphosphate 2′,3′-cyclic phosphate has been shown to be formed from guanosine 3′,5′-bisdiphosphate in crystals of stringent response factor SpoT and purine biosynthetic enzyme adenylosuccinate synthase.[20,21] Although the physiology of guanosine 5′-diphosphate 2′,3′-cyclic phosphate is not fully understood, it is possible that the compound is a substrate for PhnP. As noted previously, shared synteny is observed for *phnN* and *phnP* in bacterial *phn* operons.[12] In the few cases where *phnP* is absent, its place is taken by the *rcsF* gene, which encodes a clade (pfam06299) within the 2H-phosphodiesterase superfamily.[22] Analogous to PhnP, 2H-phosphodiesterases have activity toward ribonucleoside 2′,3′-phosphates as well as ADP-α-D-ribosyl 1′,2′-cyclic phosphate, an intermediate of tRNA splicing.[23] Synteny, therefore, suggests a biochemical link between phosphorylation of α-D-ribosyl 1,5-bisphosphate (**3b**) and hydrolysis of cyclic phosphate diesters by PhnP.

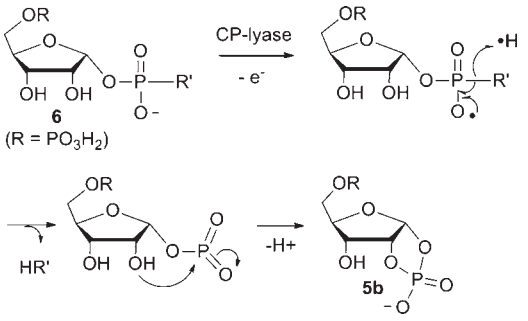
An *E. coli phnP* mutant was previously shown to accumulate two radioactive intermediates in the culture supernatant when grown in the presence of $^{32}P_i$ and unlabeled methylphosphonic acid (**1a**).[24] Addition of purified PhnP to these extracts converts one intermediate to a new product, thereby identifying the physiological substrate for the enzyme. In the present study, two intermediates were likewise observed by ^{31}P NMR when a *phnP* mutant strain was cultured with **1a** as well as a variety of other alkylphosphonates or phosphite (the exceptions being 2-aminoethylphosphonate (**1e**), which likely gives rise to an additional N-acetyl derivative,[7] and phenylphosphonic acid, which gave rise to only a single intermediate, see below). One of these intermediates was identified as α-D-ribosyl 1-ethylphosphonate (**2b**), previously described by others.[10] The other intermediate was α-D-ribosyl 1,2-cyclic phosphate (**5a**). Purified PhnP converted **5a** to α-D-ribosyl 1-phosphate (**3a**). The radioactive intermediates described above are phosphate esters. Therefore, it is likely that compounds **2b** and **5a** represent C5-dephosphorylated variants of the in vivo intermediates. This indeed appears to be the case as PhnP also regiospecifically hydrolyzes the corresponding 5-phosphate ester **5b** to **3b**, a substrate for α-D-ribosyl 1,5-bisphosphate phosphokinase. Although the previously detected radiolabeled compounds are also predominant in the growth medium, rather than in the cells, the amount of the radiolabeled compounds is much lower than that of the dephosphorylated versions detected here. The concentration of radiolabeled **5b** generated by methylphosphonic acid (**1a**)-fed cells was determined as 15 μM,[24] compared to 0.36 mM observed for **5a** (Table 1). Thus, the low amounts of the C5-phosphorylated compounds may have escaped detection by ^{31}P NMR, and reciprocally, the dephosphorylated forms would not be detected by the radiolabeling approach.

On the basis of the results of this study, we propose a hypothetical pathway for assimilation of phosphonic acid, shown in Scheme 4. The PhnP-catalyzed hydrolysis of **5b** is shown as reaction iii. We propose that **5b** is the product of CP lyase activity (reaction ii) and that the substrate for CP lyase is 5-phospho-α-D-ribosyl 1-phosphonate (**6**) (see below and Scheme 5). Due to the structural resemblance of **6** and **3b**, we furthermore suggest that the process operates as a cycle, where reaction i involves an exchange of the anomeric phosphate of **3b** with an alkylphosphonic acid to yield **6**. Since both **3b** and **6** have α-configuration at the anomeric carbon atom, reaction i would need to proceed through a double displacement mechanism involving a *β*-ribosyl intermediate that is attacked by the alkylphosphonic acid. It is therefore likely that reaction i may involve at least one additional intermediate. The enzymes catalyzing reactions i and ii may be encoded by some or all of the *phnGHIJKLM* cistrons. According to Scheme 4, *phnN*-specified α-D-ribosyl 1,5-bisphosphate phosphokinase functions as a feeder pathway for the phosphonic acid assimilation cycle by providing a starting compound **3b**, and thus, we suggest that α-D-ribosyl 1,5-bisphosphate phosphokinase is not part of the cycle. If α-D-ribosyl 1,5-bisphosphate phosphokinase and PRPP (**4**) were members of the cycle, a Δ*prs* strain, which lacks the normal pathway for PRPP (**4**) synthesis, should not be phosphonic acid growth-deficient. Or stated in another way, the synthesis of PRPP (**4**) by *prs*-specified PRPP synthase is necessary for phosphonic acid utilization. According to Scheme 4, the process catalyzed by α-D-ribosyl 1,5-bisphosphate phosphokinase is fully reversible, as all of the bonds formed and broken are phosphoric acid anhydride bonds. This may explain why PhnN can provide sufficient levels of **4** for NAD biosynthesis.[11]. The pathway for phosphonic acid catabolism described in Scheme 4 may also explain the failure hitherto to reliably observe CP lyase activity in vitro,[1] as **6** rather than a simple alkylphosphonic acid would need to be provided as a substrate. Alternatively, in vitro CP lyase activity may be demonstrated with α-D-ribosyl 1,5-bisphosphate as a substrate, as well as any cosubstrate or cofactor needed for the conversion of **3b** to **6**. Formation of methane from methylphosphonate (**1a**) has been demonstrated in vitro, although no intermediates were determined.[24a]

Scheme 4. Proposed Pathway for Phosphonic Acid Catabolism by CP Lyase



Scheme 5. Proposed Mechanism for C−P Bond Cleavage by CP Lyase



Finally, from Scheme 4 it is clear that *phnP* mutants accumulate **5b** when fed phosphonic acids, as **5b** cannot be further catabolized. The reason for accumulation of **6**, furthermore, may be explained by product inhibition of CP lyase by **5b**. Accumulation of **5b** and **6** results in dephosphorylation and excretion of dephosphorylated **5b** (i.e., **5a**) and dephosphorylated **6** (i.e., **2**), respectively, as described above. Accumulation of **2** was observed after growth of the *phnP* strain with any of the phosphonic acids analyzed with the exception of phenylphosphonic acid (**1f**), where no α-D-ribosyl 1-phenylphosphonate (**2f**) was detected. The reason for this lack of accumulation of **2f** is presently unknown. One possibility is that the amount formed was undetectable; for example, because α-D-5-phosphoribosyl 1-phenylphosphonate was not dephosphorylated or excreted. Alternatively, **5b** might not cause product inhibition of CP lyase when α-D-5-phosphoribosyl 1-phenylphosphonate was the substrate.

The conversion of **6** to the cyclic phosphate ester **5b** by CP lyase may proceed according to the radical mechanism previously suggested by the pioneering work of Frost and co-workers.[25−27] We suggest two changes to this putative mechanism. First, we suggest that **6** is the substrate for C−P bond cleavage rather than a simple alkylphosphonic acid. Central to the mechanism is oxidation of **6** to form a phosphonyl radical (Scheme 5). Rearrangement of the radical intermediate would result in C−P bond cleavage, releasing a carbon-centered radical in the case of an alkyl or phenylphosphonate ester or a hydrogen radical in the case of the phosphite ester. These radicals, presumably, are quenched by the enzyme (to form RH or H_2). Concurrently, a ribosyl 1-metaphosphate intermediate is formed. Second, we suggest that rather than direct hydrolysis to yield ribosyl 1,5-bisphosphate (**3b**), it is more likely that the high effective molarity of the neighboring C2 hydroxyl will favor intramolecular attack on the metaphosphate ester, thus forming the cyclic phosphodiester **5b**. It is tempting to speculate that PhnP evolved to convert **5b**, a "dead-end" metabolic intermediate, to **3b**. Finally, our mechanism predicts that phosphite will be oxidized by CP lyase only after incorporation into **6**. Avila and Frost[28] also suggested a reductive radical mechanism. This mechanism is less attractive as it predicts the formation of phosphite as an intermediate and subsequent oxidation of phosphite to phosphate. If this were the case, some *phn* mutants should be able to use phosphite but not phosphonic acid as a phosphate source. However, utilization of phosphite as phosphate source requires the entire *phn* operon, suggesting phosphite, like alkylphosphonates, is incorporated into an intermediate.[3]

In conclusion, we have shown that 5-phospho-α-D-ribosyl 1,2-cyclic phosphate (**5b**) is a competent substrate for PhnP, yielding α-D-ribosyl 1,5-bisphosphate (**3b**) as the product, and that both of these compounds are intermediates of the CP lyase pathway of *E. coli*. We suggest the name phosphoribosyl 1,2-cyclic phosphodiesterase (EC 3.1.4.-) for the *phnP* gene product.

EXPERIMENTAL PROCEDURES

General. PRPP and methyl-, ethyl-, propyl-, phenyl-, and aminomethylphosphonic acids were obtained from Sigma−Aldrich. 2-Aminoethylphosphonic acid was purchased from Acros Organics (Geel, Belgium), whereas phosphite was purchased from Riedel-deHaën (Buchs, Switzerland). α-D-Ribosyl 1-phosphate was prepared enzymatically by phosphorolysis of uridine with uridine phosphorylase in the presence of P_i followed by ion-exchange chromatography on a Dowex 1 column by K. F. Jensen, University of Copenhagen. PhnP was purified as previously described.[29] NMR spectra were recorded on Bruker Avance 400 or 600 MHz spectrometers. ^{1}H chemical shifts (δ) are reported relative to HDO, whereas ^{31}P chemical shifts are reported relative to a 17 mM phosphoric acid external standard. ^{1}H and ^{31}P resonances were assigned by ^{1}H/^{1}H correlation spectroscopy (COSY) and ^{1}H/^{31}P heteronuclear multiple bond correlation spectroscopy (HMBC).

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Table 3. *E. coli* Strains Used

strain	genotype	source, reference, or construction
BW17471	F$^-$ *phnE*(EcoK$^+$) *phnM28*::Tn*phoA′-1*[a]	B. L. Wanner[3]
BW26904	F$^-$ *lamB rph-1* Δ*pstS605*::*cat*	B. L. Wanner[35]
HO770	F$^-$ *deoD gsk-3 udp phnE*(EcoK$^-$)[b]	36
HO773	F$^-$ *deoD gsk-3 udp phnE*(EcoK$^-$) Δ*prs-4*::Kan^r [b]	36
HO1429	F$^-$ *deoD gsk-3 udp phnE*(EcoK$^+$)[b]	HO770, 2-aminoethylphosphonic acid as phosphate source
HO1440	F$^-$ *deoD gsk-3 udp phnE*(EcoK$^+$) Δ*prs-4*::Kan^r [b]	P1(HO773) × HO1429, Kan^r [c]
HO2531	F$^-$ *phnE15*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2533	F$^-$ *phnE*(EcoK$^+$) *phnG35*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2534	F$^-$ *phnE*(EcoK$^+$) *phnH13*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2535	F$^-$ *phnE*(EcoK$^+$) *phnI40*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2536	F$^-$ *phnE*(EcoK$^+$) *phnJ14*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2537	F$^-$ *phnE*(EcoK) Δ(*lac*)χ74 Δ*phoA532 phnK6*::Tn*5*−112 Δ*pstS605*::*cat*	9
HO2538	F$^-$ *phnE*(EcoK$^+$) *phnL39*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	9
HO2539	F$^-$ *phnE*(EcoK$^+$) *phnM28*::Tn*phoA′-1* Δ*pstS605*::*cat*[a]	P1(BW26904) × BW17471, Cml^r [c]
HO2540	F$^-$ *phnE*(EcoK$^+$) Δ*uidA5 phnN45*::Tn*phoA′-3* Δ*pstS605*::*cat*[a]	24
HO2541	F$^-$ *phnE*(EcoK$^+$) *phnO38*::Tn*phoA′-9* Δ*pstS605*::*cat*[a]	24
HO2542	F$^-$ *phnE*(EcoK$^+$) *phnP54*::Tn*phoA′-1* Δ*pstS605*::*cat*[a]	24
HO2568	F$^-$ *phnE*(EcoK$^+$) Δ*uidA5 rph-1 rpoS396*$_{am}$ Δ*pstS605*::*cat*[a]	9
HO2680	F$^-$ Δ(*lac*)χ74 Δ(*phnCDEFGHIJKLMNOP*)33−30 Δ*pstS605*::*cat*	9
HO3342	F$^-$ *deoD gsk-3 udp phnE*(EcoK$^+$) Δ*prs-4*::Kan^r Δ*pstS605*::*cat*[b]	P1(HO2568) × HO1440, Cml^r [c]

[a] Also contains Δ(*lac*)χ74 Δ*phoA532 phn-10*::*uidA2*−*aadA* Δ(*fumCA manA uidA add*). [b] Also contains *araC*$_{am}$ *araD* Δ(*lac*)U169 *trp*$_{am}$ *mal*$_{am}$ *rpsL relA thi supF*. [c] Cml^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

Bacterial Strains and Growth Conditions. The *E. coli* K-12 strains used and their origin or construction are shown in Table 3. Mutations caused by Tn*phoA′-1* are polar, whereas mutations caused by Tn*phoA′-9* are nonpolar.[3] The strains HO1440, HO2538, and HO3342 were constructed by bacteriophage P1-mediated transduction.[30] *phnE*-(EcoK$^-$) is the wild-type *E. coli* K12 *phnE* allele, which contains an 8-base-pair duplication. This results in premature termination of translation of the *phnE*-containing mRNA and phosphonic acid growth deficiency.[31] *phnE*(EcoK$^+$) is a mutant variant that has lost the 8-base-pair duplication, resulting in translation of the entire open reading frame of the *phnE* mRNA and phosphonic acid growth proficiency. Cells of *E. coli* and *Salmonella enterica* serotype Typhimurium strain F183 (*purF*) were grown at 37 °C in a low-phosphate, Tris-buffered medium, 03P,[24] containing 0.3 mM P$_i$ with glucose (0.2%) as carbon source. Guanosine was added to 30 mg·L^{-1} and uridine to 20 mg·L^{-1}, whereas histidine and tryptophan were added to 40 mg·L^{-1} each, and thiamin was added to 1 mg·L^{-1}. Cell growth was monitored as optical density (OD) at 600 nm in a cell density meter (model 40, Fischer Scientific). An OD$_{600}$ of 1 (1-cm light path) corresponds to approximately 5.5 × 10^{11} cells·L^{-1}. Chloramphenicol and kanamycin were used at 30 mg·L^{-1}. To analyze the accumulation of organophosphonic acid degradation intermediates by ^{31}P NMR, cells were grown to an OD$_{600}$ of 0.45, at which time an organophosphonic acid was added to 2 mM unless otherwise stated. After 5 h of incubation cells were removed by centrifugation, and the supernatant fluid was passed through a 0.45 μm filter. Supernatant fluids were stored at −20 °C. Solid media were buffered with 3-(*N*-morpholino)propanesulfonic acid (Mops). Glassware was washed with nitrous acid and agar was washed with water to reduce phosphate contamination.

Purification of α-D-Ribofuranosyl 1-Ethylphosphonate (2b). The supernatant fluid of a 2 L culture of strain HO2542 (*phnP*) grown with 0.3 mM **1b** was loaded on a hydroxide form of an AG1−8X column (2.5 × 19 cm) equilibrated by an Äkta fast protein liquid chromatography (FPLC) system (GE Healthcare, Canada). After the column was washed with 300 mL of deionized water, a gradient of 0−4.0 M ammonium formate in 0.1 M formic acid was used to elute α-D-ribosyl 1-ethylphosphonate (**2b**) at a flow rate of 2.0 mL·min^{-1}. Fractions containing **2b** eluted at approximately 1.5 M ammonium formate and were identified by ^{31}P NMR (δ = 30.3 ppm). The solvent was removed in vacuo to afford **2b**. ^{1}H NMR (D$_2$O, 400 MHz): δ 5.57 (H1, dd, J_{H1-H2} = 4.0 Hz, J_{H1-P} = 6.5 Hz, 1H), 4.16 (H4, dd, J_{H4-H3} = 3.4 Hz, J_{H4-H5} = 7.6 Hz, 1H), 4.07 (H2, dd, J_{H2-H1} = 4.0 Hz, J_{H2-H3} = 6.4 Hz, 1H), 4.02 (H3, dd, J_{H3-H4} = 3.4 Hz, J_{H3-H2} = 6.5 Hz, 1H), 3.7 (H5ab, m, 2H), 1.60 (CH$_2$, dd, J_{H6-H7} = 7.6 Hz, J_{H6-P} = 17 Hz, 2H), 1.02 (CH$_3$, dt, J_{H7-H6} = 7.7 Hz, J_{H7-P} = 15 Hz, 3H). ^{31}P NMR: δ 30.3 (ddd, *J* = 24, 18, and 6.2 Hz).

Synthesis of 5-Phospho-α-D-ribofuranosyl 1,2-Cyclic Phosphate (5b). A modification of a previously described procedure for conversion of **4** to 5-phosphoribosyl 1,2-cyclic phosphate (**5a**) was used.[32] PRPP (**4**) (17 mg) was dissolved in 1.0 mL of water and barium acetate was added to 5 mM. The solution was brought to pH 10.5−11.0 by addition of 1.0 M sodium hydroxide and then incubated at room temperature for 6 days. The progress of the reaction was followed by ^{31}P NMR and comparison with the previously published values for the chemical shift of the 1,2-cyclic phosphate (δ 18.6 ppm).[15,16] After complete conversion, the highly turbid solution was cleared by centrifugation and the supernatant fluid was neutralized by the addition of 0.50 M Tris-HCl, pH 7.6. NMR spectra were recorded in 25 mM Tris-HCl, pH 7.6, with 10−20% D$_2$O as a solvent lock. Protons were assigned on the basis of ^{1}H/^{1}H COSY spectra. ^{1}H NMR (600 MHz): δ 5.84 (H1, dd, J_{H1-H2} = 4.1 Hz, J_{H1-P} = 17 Hz, 1H), 4.81 (H2, m), 4.12 (H3, dd, *J* = 1.2 and 4.8 Hz, 1H), 4.09 (H4, m, 1 H), 3.83 (H5a, ddd, *J* = 12, 4.3, and 2.3 Hz, 1H), 3.96 (H5b, ddd, *J* = 11, 5.8, and 4.0 Hz). ^{31}P NMR δ 4.1 (P5, t, *J* = 5.0 Hz), 18.7 (P1,2, dd, $J_{P1,2-H1}$ = 17 Hz, $J_{P1,2-H2}$ = 4.3 Hz). To synthesize radiolabeled **5b**, radiolabeled **4** was first prepared. A culture (1 mL) of the purine auxotrophic *S. enterica* strain F183 was grown in the presence of ^{32}P$_i$ (0.75 MBq, Nex053, Perkin-Elmer, Fremont, CA) with limited hypoxanthine (3.0 mg·L^{-1}) as purine source to increase the synthesis of **4**.[33] Ninety minutes after onset of purine starvation (determined from the growth curve of a parallel, unlabeled culture), 100 μL of cold 4.0 M formic

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acid was added to the culture, which was then vigorously shaken and left on ice for 30 min, at which time debris was removed by centrifugation. The cleared extract was applied along a line 2 cm from the bottom of a 20 × 20 cm plastic sheet coated with poly(ethylenimine)−cellulose. After drying, the chromatogram was developed in 0.85 M potassium phosphate previously titrated to pH 3.4 with 0.85 M phosphoric acid. Following autoradiography, radioactive **4** was scraped off and eluted with 2 mL of 0.3 M ammonia. Unlabeled **4** was added to 2 mM and barium acetate to 0.4 mM. The reaction was incubated at room temperature for 5 days, at which time it was cleared by centrifugation and neutralized. Approximately 60% of the initial **4** was converted to the 1,2-cyclic phosphate **5b**. Procedures and solvents for TLC have been described before.[33,34] Autoradiography and quantization of radioactive material were performed with the Cyclone storage phosphor system (Perkin-Elmer).

Reaction of PhnP with 5-Phospho-α-D-ribofuranosyl 1,2-Cyclic Phosphate (5b). Purified PhnP (5 μM) was added to a solution of 10 mM **5b** in 25 mM Tris-HCl, pH 7.6, and 10−20% D_2O. The solution was incubated at 37 °C for 1 h. ^{1}H NMR (600 MHz): δ 5.53 (H1, dd, J_{H1-P} = 6.3 Hz, J_{H1-H2} = 4.1 Hz), 4.07 (H2, m, 1H), 4.04 (H3, dd, J_{H3-H4} = 3.6 Hz, J_{H2-H3} = 6.1 Hz, 1H), 4.21 (H4 dd, J_{H3-H4} = 3.9 Hz, J_{H4-H5} = 7.6 Hz, 1H), 3.76 (H5, dq, J = 11 and 5.1 Hz, 2H). ^{31}P NMR (600 MHz) δ 3.7 (P5, dd, J_{P5-H5} = 4.7 Hz), 2.3 (P1, d, J_{P1-H1} = 6.2 Hz). ^{1}H/^{31}P HMBC spectra are available as Supporting Information.

^{32}P Labeling of CP Lyase Intermediates. Labeling of CP lyase intermediates of strain HO2542 (*phnP*) with $^{32}P_i$ in the presence of unlabeled methylphosphonic acid and subsequent TLC analysis was performed as described previously.[24]

■ ASSOCIATED CONTENT

Supporting Information. Two figures showing ^{1}H/^{31}P HMBC spectra for **5b** and **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Chen, C. M.; Ye, Q. Z.; Zhu, Z. M.; Wanner, B. L.; Walsh, C. T. *J. Biol. Chem.* **1990**, *265*, 4461–4471.
(2) Metcalf, W. W.; Wanner, B. L. *Gene* **1993**, *129*, 27–32.
(3) Metcalf, W. W.; Wanner, B. L. *J. Bacteriol.* **1993**, *175*, 3430–3442.
(4) Wanner, B. L.; Metcalf, W. W. *FEMS Microbiol. Lett.* **1992**, *79*, 133–139.
(5) Rizk, S. S.; Cuneo, M. J.; Hellinga, H. W. *Protein Sci.* **2006**, *15*, 1745–1751.
(6) Gebhard, S.; Cook, G. M. *J. Bacteriol.* **2008**, *190*, 1335–1343.
(7) Errey, J. C.; Blanchard, J. S. *Biochemistry* **2006**, *45*, 3033–3039.
(8) Yakovleva, G. M.; Kim, S. K.; Wanner, B. L. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 573–578.
(9) Adams, M. A.; Luo, Y.; Hove-Jensen, B.; He, S. M.; van Staalduinen, L. M.; Zechel, D. L.; Jia, Z. *J. Bacteriol.* **2008**, *190*, 1072–1083.
(10) Avila, L. Z.; Draths, K. M.; Frost, J. W. *Biorg. Med. Chem. Lett.* **1991**, *1*, 51–54.
(11) Hove-Jensen, B.; Rosenkrantz, T. J.; Haldimann, A.; Wanner, B. L. *J. Bacteriol.* **2003**, *185*, 2793–2801.
(12) Podzelinska, K.; He, S. M.; Wathier, M.; Yakunin, A.; Proudfoot, M.; Hove-Jensen, B.; Zechel, D. L.; Jia, Z. *J. Biol. Chem.* **2009**, *284*, 17216–17226.
(13) Echols, H.; Garen, A.; Garen, S.; Torriani, A. *J. Mol. Biol.* **1961**, *3*, 425–438.
(14) Surin, B. P.; Rosenberg, H.; Cox, G. B. *J. Bacteriol.* **1985**, *161*, 189–198.
(15) Fathi, R.; Jordan, F. *J. Org. Chem.* **1988**, *53*, 1997–2001.
(16) Meola, M.; Yamen, B.; Weaver, K.; Sandwick, R. K. *J. Inorg. Biochem.* **2003**, *93*, 235–242.
(17) Hove-Jensen, B. *J. Bacteriol.* **1988**, *170*, 1148–1152.
(18) Hove-Jensen, B. *Mol. Microbiol.* **1989**, *3*, 1487–1492.
(19) Hove-Jensen, B. *J. Bacteriol.* **1996**, *178*, 714–722.
(20) Hogg, T.; Mechold, U.; Malke, H.; Cashel, M.; Hilgenfeld, R. *Cell* **2004**, *117*, 57–68.
(21) Hou, Z.; Cashel, M.; Fromm, H. J.; Honzatko, R. B. *J. Biol. Chem.* **1999**, *274*, 17505–17510.
(22) Mazumder, R.; Iyer, L. M.; Vasudevan, S.; Aravind, L. *Nucleic Acids Res.* **2002**, *30*, 5229–5243.
(23) Culver, G. M.; Consaul, S. A.; Tycowski, K. T.; Filipowicz, W.; Phizicky, E. M. *J. Biol. Chem.* **1994**, *269*, 24928–24934.
(24) (a) Kononova, S. V.; Trutko, S. M.; Laurinavichus, K. S. *Appl. Biochem. Microbiol.* **2007**, *43*, 394–398. (b) Hove-Jensen, B.; Rosenkrantz, T. J.; Zechel, D. L.; Willemoes, M. *J. Bacteriol.* **2010**, *192*, 370–374.
(25) Avila, L. Z.; Frost, J. W. *J. Am. Chem. Soc.* **1988**, *110*, 7904–7906.
(26) Cordeiro, M. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 332–334.
(27) Frost, J. W.; Loo, S.; Cordeiro, M. L.; Li, D. *J. Am. Chem. Soc.* **1987**, *109*, 2166–2171.
(28) Avila, L. Z.; Frost, J. W. *J. Am. Chem. Soc.* **1989**, *111*, 8969–8970.
(29) Podzelinska, K.; He, S.; Soares, A.; Zechel, D. L.; Hove-Jensen, B.; Jia, Z. *Acta. Crystallogr. Sect. F: Struct. Biol. Cryst. Commun.* **2008**, *64*, 554–557.
(30) Silhavy, T.; Berman, M.; Enquist, L. *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1984.
(31) Iqbal, S.; Parker, G.; Davidson, H.; Moslehi-Rahmani, E.; Robson, R. L. *J. Bacteriol.* **2004**, *186*, 6118–6123.
(32) Khorana, H. G.; Fernandes, J. F.; Kornberg, A. *J. Biol. Chem.* **1958**, *230*, 941–948.
(33) Jensen, K. F.; Houlberg, U.; Nygaard, P. *Anal. Biochem.* **1979**, *98*, 254–263.
(34) Houlberg, U.; Hove-Jensen, B.; Jochimsen, B.; Nygaard, P. *J. Bacteriol.* **1983**, *154*, 1485–1488.
(35) Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640–6645.
(36) Post, D. A.; Switzer, R. L.; Hove-Jensen, B. *Microbiology* **1996**, *142* (Pt 2), 359–365.