Catabolism and Detoxification of 1-Aminoalkylphosphonic Acids: N-Acetylation by the phnO Gene Product

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Abstract

In *Escherichia coli* uptake and catabolism of organophosphonates are governed by the *phnCDEFGHIJKLMNOP* operon. The *phnO* cistron is shown to encode aminoalkylphosphonic *N*-acetyltransferase, which utilizes acetylcoenzyme A as acetyl donor andaminomethylphosphonate, (S)- and (R)-1-aminoethyolphosphonate, 2-aminoethyl- and 3-aminoethyolphosphonate as acetyl acceptors. Aminomethylphosphonate, (S)-1-aminoethyolphosphonate, 2-aminoethyl- and 3-aminoethyolphosphonate are used as phosphate source by *E. coli* *phn* strains. 2-Aminoethyl- or 3-aminoethyolphosphonate but not aminomethylphosphonate or (S)-1-aminoethyolphosphonate is used as phosphate source by *phnO* strains. Neither *phn* nor *phnO* strains can use (R)-1-aminoethyolphosphonate as phosphate source. Utilization of aminomethylphosphonate, (S)-1-aminoethyolphosphonate requires the expression of *phnO*. In the absence of *phnO*-expression (S)-1-aminoethyolphosphonate is bacteriocidal and rescue of *phnO* strains requires the simultaneous addition of α-alanine and phosphate. An intermediate of the carbon-phosphorus lyase pathway, 5′-phospho-α-α-d-ribosyl 1′- (2-N-acetamidoethyolphosphonate), a substrate for carbon-phosphorus lyase, was found to accumulate in cultures of a *phnP* mutant strain. The data show that the physiological role of *N*-acetylation by *phnO*-specified aminoalkylphosphonate *N*-acetyltransferase is to detoxify (S)-1-aminoethyolphosphonate, an analog of α-alanine, and to prepare (S)-1-aminoethyolphosphonate and aminomethylphosphonate for utilization of the phosphorus-containing moiety.

Introduction

*Escherichia coli* as well as many other bacterial species are able to use a number of phosphorus-containing compounds as phosphate source. The preferred source is inorganic phosphate ion (P$_i$). When P$_i$ is low or absent, expression of the operons of the Pho regulon is derepressed and other phosphate sources may be utilized. Thus, phosphate esters and organophosphonates also are P$_i$ sources for *E. coli*. Utilization of organophosphonates as P$_i$ source by *E. coli* requires the 14-cistron *phnCDEFGHIJKLMNOP* operon, which is a member of the Pho regulon, and which specifies the carbon-phosphorus (C–P) lyase pathway [1,2]. *E. coli* is capable of utilizing both alkylphosphonates, such as methyl-(MePn), ethyl-(EtPn) and propylphosphonate (PrPn) as well as aminoalkylphosphonates, such as aminomethyl- (AmMePn), 2-aminoethyl- (2AmEtPn) and 3-aminoethyolphosphonate (3AmPrPn) as P$_i$ source. Utilization of both types of phosphate is dependent on an ATP-binding cassette transport system (encoded by *phnP* [1,3], as well as two additional apparent nucleotide-binding domains for organophosphonate transport (encoded by *phnK* and *phnE* [1], the enzymes C–P lyase (encoded by *phnJ*) and presumbable also *phnGHII* [1,4], *PhnM* [5], α-α-ribosyl 1,5-diphosphophosphokinase (encoded by *phnV* [6] and phosphoribosyl cyclic phosphodiesterase (encoded by *phnP* [7]). The *phnP* cistron likely encodes a repressor of *phn* operon expression [8]. *E. coli* *phnO* may specify an enzyme with aminomethylphosphonate *N*-acetyltransferase activity as evaluated by amino acid sequence similarity with the *phnO* gene product of *Salmonella enterica* serotype Typhimurium [9]. The physiological function of *S. enterica* aminomethylphosphonate *N*-acetyltransferase is presently unknown as this organism does not contain C–P lyase. In contrast, *S. enterica* contains the enzyme phosphonoacetaldehyde phosphohydrolase, which is encoded by the *phnX* gene [10], and which is responsible for the catabolism of 2AmEtPn.

The catabolism of phosphonates by the C–P lyase pathway involves a number of enzymatic activities and intermediates the latter of which are ribose derivatives analogous those shown in Figure 1 [7,11,12]. *In vitro* analysis has shown that initially a phosphate moiety displaces adenine of ATP with the formation of a phosphate ester and with inversion of the configuration of the anomeric carbon. The product is 5′-triphospho-α-α-ribosyl 1′-phosphate and the reaction presumably is catalyzed by PhnI, and, in some way assisted by PhnG, PhnH and PhnK or PhnL. Next the α,β-diphosphoryl bond of 5′-triphospho-α-α-ribosyl 1′-phosphate is hydrolyzed by PhnM to generate 5′-phospho-α-α-ribosyl 1′-phosphate [5]. The latter compound is the substrate for C–P lyase encoded by *phnJ*, the product being 5-phospho-α-α-ribosyl 1,2-cyclic phosphate. Polypeptides other than PhnI, such as PhnG, PhnH, PhnI, PhnK or PhnL, may participate in the C–P bond cleavage as well [5,7]. 5-Phospho-α-α-ribosyl 1,2-cyclic phosphate is then hydrolyzed to form α-α-ribosyl 1,5-bisphosphate, a reaction catalyzed by *phnP*-specified phosphoribosyl cyclic phosphodiesterase [7]. The fate of the phosphate-derived phosphorus (i.e. that of the 1-phosphate of α-α-ribosyl 1,5-
N-Aminoalkylphosphonate Acetylation by \textit{phnO}

The growth response to various organophosphonates of strains containing the \textit{phnO38} or \textit{Aphn0789} alleles was compared to that of \textit{pht} and \textit{Aphn} strains (Table 1). A \textit{pht} strain (HO3414) utilized AmMePn, 2AmEtPn and 3AmPnPn as well as MePn, EtPn and PrPn as P source. The two \textit{phnO} strains utilized 2AmEtPn and 3AmPnPn as well as MePn, EtPn and PrPn as \textit{P} source, whereas they were unable to utilize AmMePn. Although the two \textit{phnO} strains qualitatively responded similarly to the various phosphonates, the growth of strain HO3413 (\textit{Aphn0789}) in general was poorer than that of strain BW17572 (\textit{phnO38}). AmMePn may be regarded as an analog of glycine and could be expected to take the place of glycine in one or more biochemical reactions. However, the addition of glycine to AmMePn-containing medium did not restore growth of the \textit{phnO} strains (not shown). In contrast, the addition of P to AmMePn-containing medium restored growth. A \textit{Aphn} strain (HO2678) as expected was unable to utilize any of the phosphonates tested. We conclude from these observations that the \textit{phnO}-specified aminoalkylphosphonate \textit{N-acetyltransferase} is obligatory for growth with AmMePn as \textit{P} source, but not for growth with the other aminoalkylphosphonates 2AmEtPn and 3AmPnPn, nor for growth with alkylphosphonates as \textit{P} source.

The utilization of the \textit{S} and \textit{R} enantiomers of 1-aminoethylphosphonate was also analyzed. To test if S1AmEtPn was used as a \textit{P} source, special conditions were employed. This compound was found to be conditionally bacteriocidal for several of the strains used, as cell lysis was initiated approximately 20 min upon addition of the compound to cell suspensions. S1AmEtPn is an analog of \textit{d}-alanine, a precursor of bacterial peptidoglycan biosynthesis, and as such is a competitive inhibitor of alanine racemase [14]. To overcome this inhibitory effect, cultures of cells grown with S1AmEtPn were supplemented with \textit{d}-alanine (or in some cases \textit{l,l}-alanine), which rescued the cells from lysis. Cell lysis, therefore, was caused by inhibition of cell wall synthesis. A typical growth curve of strain HO2568 (\textit{phnO Apts}) grown with or without \textit{l,l}-alanine is shown in Figure S1. The addition of S1AmEtPn caused rapid lysis of the culture without \textit{l,l}-alanine, whereas the addition of S1AmEtPn appeared to induce only a temporary arrest in growth of the culture with \textit{l,l}-alanine present. Interestingly, strain HO2680 (\textit{Aphn Apts}) also lysed in the presence of S1AmEtPn (not shown). HO2680 is unable to transport phosphonate by the cognate phosphonate transport system, suggesting that at least under these conditions the compound was taken up by a different transport system, which was not the high affinity P transporter system specified by \textit{pstSCAB}, as this was also deleted in the strain. The growth response of the various strains to 1-aminoalkylphosphonates in solid medium is shown in Table 2. None of the strains grew with S1AmEtPn as sole supplement. If \textit{P} was added in addition to S1AmEtPn the \textit{phnO} strain grew, whereas the \textit{phnO} strains (and also the \textit{Aphn} strain) formed very small colonies, which appeared heterogeneous in colony size and morphology, thus, demonstrating the toxic effect of S1AmEtPn described above. When \textit{P} was supplied none of the strains needed to utilize S1AmEtPn. However, the wild-type strain was able to acetylate S1AmEtPn, and, thus, presumably detoxify the compound, whereas the \textit{phnO} strains were unable to acetylate and detoxify the compound, and, consequently, no or poor growth occurred. When \textit{l}-alanine was added in addition to S1AmEtPn the \textit{phnO} strain grew, whereas the \textit{phnO} strains did not. Here \textit{l}-alanine competed with its analog S1AmEtPn and inhibition was overcome. In contrast, the \textit{phnO} strains were unable to grow even
though d-alanine presumably also overcame the inhibition by S1AmEtPn in these strains. Therefore, the reason for lack of growth of the \( \text{phnO} \) strains was a lack of acetylation, and, thus, acetylation is a requisite also for catabolism of S1AmEtPn. With addition of both D-alanine and Pi, in addition to S1AmEtPn, the \( \text{phnO} \) strains resumed growth. Again D-alanine overcame the inhibition by S1AmEtPn, and the presence of Pi made the presence of S1AmEtPn redundant as Pi source. The reason for lack of growth of strain HO3414 (\( \text{phn}^+ \)) with S1AmEtPn as sole supplement is presently not clear. Constitutive expression of the \( \text{phn} \) operon, and, thus, of the \( \text{phnO} \) cistron was not sufficient to obtain growth with S1AmEtPn as sole Pi-source, as strain HO3417 (\( \text{phn}^+ \text{DpstS} \)) responded similarly to strain HO3414 (\( \text{phn}^+ \text{DpstS} \)).

The effect on cell growth of R1AmEtPn was much less dramatic. Although R1AmEtPn inhibited the growth of \( \text{phn}^+ \) strains, such as HO2568 and HO3414, no lysis was observed. This inhibition could not be alleviated neither by the addition of L-alanine, of which R1AmEtPn may be an analog, nor by the addition of D-alanine or both. Neither the \( \text{phn} \) nor the \( \text{phnO} \) strains utilized R1AmEtPn as Pi source. Furthermore, all of the \( \text{phn}^+ \) and \( \text{phnO} \) strains grew when Pi was added together with R1AmEtPn, showing that R1AmEtPn probably did not exert any severe toxic effect. We conclude from these observations that R1AmEtPn is not a Pi-source for \( \text{E. coli} \). This observation may be consistent with R1AmEtPn being a poor substrate for \( \text{phnO} \)-specified aminoalkylphosphonate \( N \)-acetyltransferase.

Accumulation of 2AmEtPn catabolic-intermediates in cultures of \( \text{E. coli} \) \( \text{phn} \) mutant strains

The \( \text{phn} \) mutant strains employed in this analysis contained the \( \text{ApS605} \) allele, which served to render \( \text{phn} \) operon expression constitutive and, thus, independent of the phosphate supply. The conversion of aminoalkylphosphonate and the accumulation of intermediates of aminoalkylphosphonate catabolism were analyzed by \( ^{31} \text{P} \) NMR spectroscopy of the growth medium, which also contained Pi, as several of the \( \text{phn} \) mutants used were unable to use phosphonate as phosphate source. A \( \text{phn}^+ \) \( \text{ApS605} \) strain (HO2568) was grown in the presence of 2AmEtPn. Figure 3A shows a \( ^{31} \text{P} \) NMR spectrum of the culture medium immediately after the addition of 2AmEtPn (\( \delta \) 16.8 ppm), whereas Figure 3B shows a \( ^{31} \text{P} \) NMR spectrum of the culture medium after 20 h of incubation at 37°C. Here the \( \delta \) 16.8 ppm-peak is greatly diminished, and, in addition, two additional peaks appear (\( \delta \) 19.5 and 23.6 ppm). The two compounds were 2NAcAmEtPn (19.5 ppm) and \( \alpha \)-D-ribosyl 1\(^\prime\)-\( N \)-acetamidoethylphosphonate (Rib1\(^\prime\)N2AcEtPn) (23.6 ppm). The time course is shown in

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**Figure 1. Catabolism of AmMePn.** Compounds: 1, NAcAmMePn; 2, S\(^\prime\)-tripospho-\( \alpha \)-\( \delta \)-ribosyl 1\(^\prime\)-\( N \)-acetamidomethylphosphonate; 3, S\(^\prime\)PRib1\(^\prime\) NAcAmMePn; 4, N-methylacetamide; 5, SPRib1\(^\prime\)2cP; 6, \( \alpha \)-\( \delta \)-ribosyl 1,5-bisphosphate; 7, PRPP; 8, diphosphate. Reactions are indicated by their enzymes: PhnO, aminoalkylphosphonate \( N \)-acetyltransferase; PhnI*, an enzyme complex where PhnI plays a crucial catalytic role, and which may involve also PhnG, PhnH, PhnJ, PhnK and/or PhnL; PhnM, S\(^\prime\)-tripospho-\( \alpha \)-\( \delta \)-ribosyl 1\(^\prime\)-phosphonate diprophosphohydrolase; PhnJ*, \( \delta \)-adenosylmethionine dependent carbon-phosphorus lyase. PhnI* may constitute a protein complex containing also PhnG, PhnH, PhnI, PhnK and/or PhnL. PhnI* and PhnJ* may be the same protein complex; PhnP, \( \text{phn} \)P specified phosphoribosyl cyclic phosphodiesterase; PhnN, \( \text{phnN} \) specified aminoalkylphosphonate phosphokinase; APRTase, \( \text{apt} \) specified adenine phosphoribosyltransferase; Ppa, ppa specified inorganic diphosphate hydrolase. The enzymes of the latter two reactions are not specified by the \( \text{phn} \) operon. APRTase is arbitrarily chosen among the 10 phosphoribosyltransferases of \( \text{E. coli} \) [21]. Any of these 10 enzymes may participate in the process. The pathway is established on the basis of refs. 4, 5, 6, 7, 11 and 13, as well as results of the present work.

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We furthermore analyzed the fate of 2AmEtPn in the culture medium of a \textit{phn} \textit{DphoS} strain (HO2542), Figure 4B. Here 2AmEtPn was converted to three compounds, two of which were the same as those formed by the \textit{phn} \textit{A} strain, δ 19.5 and 23.6 ppm, and a third compound with δ 18.6 ppm. The latter peak was caused by Rib1,2cP, which is a prominent phosphorus-catabolite immediate of \textit{phn} \textit{P} strains [7]. Analysis of a \textit{phn} \textit{DphoS} strain (HO2536) revealed a pattern similar to that of the \textit{phn} \textit{A} strain, except that Rib1,2cP was not formed as evidenced by a lack of the δ 18.6 ppm-peak, which was expected as the \textit{phn} \textit{J} gene product exerts its function (C–P bond cleavage) before that of the \textit{phn} \textit{P} gene product (cyclic phosphodiester hydrolysis) [7], Figure 4C. With strains HO2542 and HO2536 there was no loss of phosphonate phosphorus to the cells, which was also expected, as these strains are unable to convert phosphonate to a usable phosphorus-containing compound.

**Chemical structure of the 19.5 ppm-compound, 2NAcAmEtPn**

The supernatant of a 2AmEtPn-grown culture of strain HO2542 (\textit{phnP}) was applied to an ion-exchange column, and the various phosphorus-containing compounds were separated (Materials and Methods). Elution was followed by \textit{31}P NMR spectroscopy, Figure S2A. The structure of the compound responsible for the δ 19.5 ppm-peak was shown by \textit{1}H, \textit{13}C and \textit{31}P NMR spectroscopy to be 2NAcAmEtPn. The following signals were obtained: \textit{1}H NMR (400 MHz, D\textsubscript{2}O) δ ppm 3.35 (doublet (d) of triplets (t), coupling constant (\textit{J} = 7.6, 7.6, 10.2 Hz, 2H), 1.96 (singlet (s), 3H), 1.82 (multiplet (m), \textit{J} = 7.6, 7.6, 16.1 Hz, 2H) (Figure 5A); \textit{13}C NMR (101 MHz, D\textsubscript{2}O) δ ppm 173.89 (s, 1CO), 35.04 (s, 1CH\textsubscript{2}), 27.85 (d, \textit{J}_{C\text{CH}} = 131 Hz, 1CH\textsubscript{3}), 22.03 (s, 1CH\textsubscript{3}) (Figure 5B). Protons were assigned to carbons by \textit{1}H/\textit{13}C HSQC NMR (Figure 5C), and by \textit{1}H/\textit{13}C HMBC NMR (Figure 5D). \textit{1}H/\textit{13}C correlations from HSQC and HMBC NMR spectra are shown in Figure 6A. Furthermore, 2NAcAmEtPn was also the product of aminoalkylphosphonate \textit{N}-acetyltransferase activity with 2AmEtPn as substrate. This was demonstrated by mixing the two compounds (\textit{i.e.} reaction product and supernatant fluid), which resulted in enlargement of a single peak (\textit{31}P NMR, δ 19.5 ppm) rather than formation of two individual peaks (data not shown).

**Table 1. Growth response of \textit{phnO} strains to various aminoalkyl- and alkylphosphonates.**

| Strain   | Lesion | Growth with phosphate source* |
|----------|--------|------------------------------|
|          | None   | AmMePn | AmMePn+P<sub>A</sub> | 2AmEtPn | 3AmPrPn | MePn | EtPn | PrPn | P<sub>i</sub> |
| BW17572  | \textit{phnO38} | –      | –              | +++     | ++      | +++  | ++  | +    | +++          |
| HO3413   | \textit{dphnO789} | –      | ++             | ++      | +       | ++   | ++  | +    | +++          |
| HO3414   | \textit{phn} \textit{R} | –      | ++             | ++      | ++      | ++   | ++  | +    | +++          |
| HO2578   | \textit{dphn33-30} | –      | –              | –       | –       | –    | –   | –    | –/+++        |

*Growth was recorded after 48 h of incubation at 37°C: –, no growth; + and ++, intermediate growth; +++, normal (wild-type-like) growth. Phosphorus-containing compounds were added at a concentration of 0.3 mM.

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Accumulation of AmMePn catabolic-intermediates in cultures of E. coli phn mutant strains

The conversion of AmMePn was also analyzed. In general, the same pattern emerged. AmMePn was acetylated and further catabolized by the phn+, the phnJ and the phnP strain, and compounds with chemical shifts $\delta$ 13.9 and 17.4 ppm appeared in cultures of all three strains (in addition to residual AmMePn of $\delta$ 9.2 ppm). The two new compounds were N-acetamidomethylphosphonate (NAcAmMePn) (13.9 ppm) and $\alpha$-D-ribosyl 1-$(\text{N-}(\text{acetamidomethylphosphonate})$ (Rib1,N2AcAmMePn) (17.4 ppm). As expected the compound of $\delta$ 18.6 ppm (Rib1,2cP) also appeared in the AmMePn-fed cells of the phnP strain. The response, however, was less dramatic than that with 2AmEtPn. As with 2AmEtPn, there was no acetylation at all of AmMePn by the phnO strains, Figure 7, Table 3. The response of strains HO2542 (phnP DpstS) and HO2536 (phnJ DpstS) is shown in Figure S3 and S4, respectively. The more sluggish disappearance of AmMePn, as compared to that of 2AmEtPn, is consistent with the poorer growth of strain HO2568 (phn+) with AmMePn as $P_i$ source compared to that with 2AmEtPn (Table 1).

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The accumulation of compounds in the used medium after growth of the four strains HO2568 (phn+ DpstS), HO2541 (phnO

Figure 4. Time course of conversion of 2AmEtPn by phn+, phnO, phnP and phnJ strains. Symbols: squares, 2AmEtPn ($\delta$ 16.8 ppm); diamonds, 2NAcAmEtPn ($\delta$ 19.4 ppm); triangles, Rib1,N2AcAmEtPn ($\delta$ 23.6 ppm); circles, Rib1,2cP ($\delta$ 18.6 ppm). (A) Strain HO2568 (phn+ DpstS) (closed symbols) and strain HO2541 (phnO38 DpstS) (open symbol); (B) strain HO2542 (phnP DpstS); (C) strain HO2536 (phnJ DpstS).

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Table 2. Growth response of phnO strains to 0.3 mM 1-aminoethylphosphonate.

| Growth with 2AmEtPn and additive(s) | R1AmEtPn ± Pi |
|------------------------------------|---------------|
| Strain | Lesion | None | Pi | d-Alanine | P1o-alanine | –Pi | +Pi |
| BW17572 phnO38 | – | – | – | +++ | – | +++ |
| HO3413 $\Delta$phnO738 | – | – | – | +++ | – | +++ |
| HO3414 $\Delta$phn | +++ | + | +++ | – | +++ |
| HO2678 $\Delta$phn | – | – | + | +++ | – | +++ |

Growth conditions and recording were those described in Table 1. The concentration of d-alanine was 100 mg L$^{-1}$.

Few very small colonies, heterogeneous in size and morphology.

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Figure 3. Conversion of 2AmEtPn by a phn+ strain analyzed by $^{31}$P NMR. Cells of strain HO2568 (phn+ DpstS) were grown in 03P medium in the presence of 2AmEtPn and supernatant fluids were analyzed as described in Materials and Methods. (A) $^{31}$P NMR spectrum of culture supernatant immediately after addition of 2AmEtPn. Chemical shifts: 16.8 ppm, 2AmEtPn; 2.3 ppm Pi. (B) $^{31}$P NMR spectrum of culture supernatant after 20 h of incubation with 2AmEtPn. Chemical shifts: 23.6 ppm, Rib1,N2AcAmEtPn; 19.4 ppm, 2NAcAmEtPn; 2.3 ppm Pi. The peak at 0.0 ppm represents the external standard.

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Various organophosphonates.

Sometimes showed small differences among otherwise similar cultures. 

| Strain       | Lesion | Addition | Chemical shift (ppm) | Assignment |
|--------------|--------|----------|----------------------|------------|
| HO2568      | phn    | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 13.9                 | NAcAmMePn  |
|             |        |          | 17.4                 | Rib1’ NAcAmMePn |
| 2AmEtPn     |        |          | 16.8                 | 2AmEtPn    |
| R1AmEtPn    |        |          | 12.6                 | R1AmEtPn   |
| S1AmEtPn    |        |          | 12.6                 | S1AmEtPn   |
| HO2541      | phnO38 | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 20.6                 | Rib1’ NAcAmEtPn |
|             |        |          | EtPn                 | EtPn       |
|             |        |          | MePn                 | MePn       |
|             |        |          | PrPn                 | PrPn       |
| HO2541      | phnQ38 | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 16.8–17.0            | 2AmEtPn    |
| S1AmEtPn    |        |          | 12.6                 | S1AmEtPn   |
| HO2542      | phnP   | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 13.9–14.0            | NAcAmMePn  |
|             |        |          | 17.4                 | Rib1’ NAcAmMePn |
|             |        |          | 18.6                 | Rib1,2cP   |
| 2AmEtPn     |        |          | 16.8–17.1            | 2AmEtPn    |
| R1AmEtPn    |        |          | 12.6                 | R1AmEtPn   |
| S1AmEtPn    |        |          | 12.6                 | S1AmEtPn   |
| HO2536      | phnJ   | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 13.9                 | NAcAmMePn  |
|             |        |          | 17.4                 | Rib1’ NAcAmMePn |
|             |        |          | 18.6                 | Rib1,2cP   |
| 2AmEtPn     |        |          | 16.9–17.0            | 2AmEtPn    |
| EtPn        |        |          | 27.6                 | EtPn       |
| HO2536      | phnJ   | AmMePn   | 9.2                  | AmMePn     |
|             |        |          | 13.9                 | NAcAmMePn  |
|             |        |          | 17.4                 | Rib1’ NAcAmMePn |
| 2AmEtPn     |        |          | 16.9–17.0            | 2AmEtPn    |
| EtPn        |        |          | 27.6                 | EtPn       |

*Data are from samples taken after 20 h of incubation in the presence of phosphate. Due to uneven acidification of the growth media chemical shifts occasionally showed small differences among otherwise similar cultures.

Table 3. Summary of 31P NMR chemical shifts observed in culture supernatants of various *phn* strains grown with various organophosphonates.

$\Delta$psS, HO2536 (phnJ ΔpsS), and HO2542 (phnP ΔpsS) in the presence of EtPn for 20 h was also analyzed. Strains HO2536 and HO2542 contained a compound with a chemical shift δ 30.3 ppm in addition to remaining EtPn (δ 27.6 ppm), whereas there were no phosphorus-containing compounds other than EtPn in the supernatant fluids of strains HO2568 and HO2541. The δ 30.3 ppm-compound has been previously detected and determined as $\alpha$-D-ribose 1’-ethyolphosphonic acid (Rib1’ EtPn) [7]. As expected, the *phnP* strain also accumulated Rib1,2cP, a substrate of *phnP*-specified phosphoribosyl cyclic phosphodiesterase (Table 3) [7].

Although *phnO* strains readily acetylated 2AmEtPn to form 2NAcAmEtPn the process apparently was not necessary for catabolism of 2AmEtPn as demonstrated by the ability of *phnO* mutant strains to utilize 2AmEtPn as a Pi source (Table 1). A parallel process by which 2-aminoalkylphosphonate is catabolized in the absence of acetylation was also demonstrated, as the *phnO*789-harboring strain (HO3412) accumulated Rib1,2cP when fed 2AmEtPn (Table 3). In contrast, when strain HO3412 was fed AmMePn no Rib1,2cP could be detected in the culture supernatant, providing support for the suggestion that the catabolism of 1-aminoalkylphosphonates requires acetylation.

Accumulation of 5’PRib1’2NAcAmEtPn in the culture of a 2AmEtPn-grown *phnP* strain

During the purification of 2NAcAmEtPn by ion-exchange chromatography we noticed the elution of a compound with chemical shifts δ 24 and 3.6 ppm by 31P NMR spectroscopy. The chemical shift values suggested a structure containing two phosphorus atoms, one of which was bound to carbon (δ 24 ppm) the other being a phosphate ester (δ 3.6 ppm) (Figure S2B). Selected fractions were concentrated and characterized by 1H NMR spectroscopy. Although the preparation also contained 2NAcAmEtPn, we unequivocally identified the presence of 5’PRib1’2NAcAmEtPn. Protons were assigned to carbons on the basis of 1H/1H correlation spectroscopy (COSY), 13C, 1H-distortions enhancement by polarization transfer (DEPT) NMR, 1H/13C HSQC and 1H/13C HMBC NMR spectra as well as 1H/31P HMBC NMR spectra. The following signals were obtained: 1H NMR (600 MHz, D2O): δ 5.73 (H1’, m, 1H), 4.41 (H4’, m, 1H), 4.24 (H2’/H3’, m, 3H), 4.04 (H5’, m, 2H), 3.45 (H2 of 5’PRib1’2NAcAmEtPn and 2NAcAmEtPn, m, 4H), 2.04 (H4 of 5’PRib1’2NAcAmEtPn and 2NAcAmEtPn, m, 3H), 1.89 (H1 of 5’PRib1’2NAcAmEtPn and 2NAcAmEtPn as well as a contaminating compound, m, 7H) (Figure S8A); 13C NMR (125 MHz, D2O) δ 173.9 (s, CO, C5), 97.4 (d, $J_{C\alpha}$ = 5.8 Hz, CH, C1’), 84.3 (d, $J_{C\alpha}$ = 8.5 Hz, CH, C4’), 71.3 (s, CH, C2’), 69.4
(s, CH, C3'), 64.7 (d, JCP = 4.6 Hz, CH2, C5'), 34.6 (s, CH2, C2), 30.6 (d, JCP = 137 Hz, CH2, C1), 22.0 (s, CH3, C4) (Figure 8B); 31P NMR (400 MHz, D2O) δ 23.7 (P1) and 0.55 (P5), in addition to 21.7 ppm (P of 2NAcAmEtPn) and 0.17 ppm P. The two-dimensional NMR spectra of 5'PRib1'2NAcAmEtPn are shown in Figure 8C (1H/13C HSQC spectrum) and Figure 8D (1H/31P HMBC spectrum) as well as Figure S5 (1H/1H COSY spectrum) and Figure S6 (1H/13C HMBC spectrum). A list of the chemical

![Figure 5](https://example.com/fig5.png)

**Figure 5. Characterization by NMR of 2NAcAmEtPn.** Spectra: A, 1H NMR; B, 31C NMR; C, 1H/13C HSQC NMR; D, 1H/13C HMBC NMR.
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![Figure 6](https://example.com/fig6.png)

**Figure 6.** Observed 1H/13C HSQC, 1H/13C HMBC and 1H/31P HMBC correlations of 2NAcAmEtPn and 5'PRib1'2NAcAmEtPn. Black arrows: correlations observed by 1H/13C HSQC and 1H/13C HMBC spectroscopy; blue arrows: correlations observed by 1H/31P HMBC spectroscopy. A, 2NAcAmEtPn; B, 5'PRib1'2NAcAmEtPn.
doi:10.1371/journal.pone.0046416.g006

![Figure 7](https://example.com/fig7.png)

**Figure 7.** Time course of conversion of AmMePn by strain HO2568 (phn) (closed symbols) and strain HO2541 (phnO38) (open symbol). Symbols: squares, AmMePn (δ 9.2 ppm); diamonds, NAcAmMePn (δ 13.9 ppm); triangles, Rib1'NAcAmMePn (δ 17.4 ppm).
doi:10.1371/journal.pone.0046416.g007
shifts of individual protons and their correlation with carbon and phosphorus atoms is given in Table 4. Observed ¹H/¹³C correlations from HSQC and HMBC NMR spectra as well as ¹H/³¹P correlations from HMBC NMR spectra are shown in Figure 6B. Finally, analysis by electrospray ionization mass spectrometry (ESI-MS) (negative ion mode) revealed a molecular ion peak at \( m/z = 378.0351 \) corresponding to the expected value for the molecular ion of \( 5'PRib1,2NAcAmEtPn \) (C₉H₁₈NO₁₁P₂) (378.0355). Another compound (\( d_{24} \) ppm) was also observed eluting 50 to 97 mL. This compound was not characterized further but is believed to be \( Rib1,2cP \), i.e. a 5'-dephosphorylated derivative of \( 5'PRib1,2NAcAmEtPn \) [7].

Polarity of the \( \Delta phnO789 \) allele on \( phnP \) gene expression

During genetic manipulations we noted that the utilization of alkylphosphonates or aminoalkylphosphonates other than AmMePn as P₇ source was considerably retarded in strains harboring the \( \Delta phnO789:kan \) allele compared to strains harboring the \( \Delta phnO38::TnphoA-9\) or wild-type \( phnO \) alleles. The three strains were isogenic as they differed only with respect to the \( phnO \) alleles. Nevertheless, strain HO3413 (\( \Delta phnO789 \)) consistently formed smaller colonies on solid medium than did strains HO3414 (\( phnO \)) or HO3418 (\( phnO38 \)). This behavior is also evident from the data of Table 1. Compare for example the growth response of strain BW17572 (\( phnO \)) with that of strain HO3413 (\( \Delta phnO789 \)) on 2AmEtPn as P₇ source. Additionally, the doubling times in medium containing MePn, which is catabolized without the participation of the \( phnO \) gene product, as P₇ source were 180, 160 and 300 min for HO3414 (\( phnO \)), HO3418 (\( phnO38 \)) and HO3413 (\( \Delta phnO789 \)), respectively. Insight to the basis of these differences was gained, when a 2AmEtPn-fed culture of strain HO3412 (\( \Delta phnO789 \Delta pstS \)) was observed to accumulate Rib1,2cP (\( 31P \) NMR, \( \delta = 18.6 \) ppm, Table 3) to an amount of approximately 15% of the remaining 2AmEtPn. In contrast, a 2AmEtPn-fed culture of strain HO2541 (\( phnO38 \Delta pstS \)) did not accumulate Rib1,2cP. Rib1,2cP is a substrate of \( phnP \)-specified phosphoribosyl cyclic phosphodiesterase [7]. Thus, the phosphonate-bradytrophic (i.e. slow-growing) phenotype of \( \Delta phnO789:kan \)-harboring strains is ascribed to insufficient activity of \( phnP \)-specified phosphoribosyl cyclic phosphodiesterase due to polarity of the \( \Delta phnO789 \) allele on the expression of the downstream \( phnP \) cistron.

Figure 8. Characterization by NMR of 5'PRib1,2NAcAmEtPn. Protons, carbons and phosphorus of 2NAcAmEtPn are labeled in red. Spectra: A, \(^1\)H NMR; B, \(^{13}\)C NMR; C, \(^1\)H/\(^{13}\)C HSQC NMR; D, \(^1\)H/\(^{31}\)P HMBC NMR. doi:10.1371/journal.pone.0046416.g008
Table 4. Assignment of the NMR spectroscopic signals observed for 5’PRib1’2NAcAmEtPn.a

| C   | 13C NMR (ppm) | 1H NMR (ppm, multiplicity, integration) | 1H/13C HMBC (ppm) | 1H/31P HMBC (ppm) |
|-----|---------------|----------------------------------------|-------------------|-------------------|
| 1   | 30.6          | 1.89, m, 7b                             | 23.5, 21.5        |                   |
| 2   | 34.6          | 3.45, m, 4                              | 23.5, 21.6        |                   |
| 3   | 173.9         |                                        |                   |                   |
| 4   | 22.0          | 2.04, s, 5                              | 172.1, 173.9      |                   |
| 1’  | 97.4          | 5.73, m, 1                              | 23.5              |                   |
| 2’  | 71.3          | 4.24, m, 3’                             |                   |                   |
| 3’  | 69.4          | 4.24, m, 3’                             |                   |                   |
| 4’  | 84.3          | 4.41, m, 1                              |                   |                   |
| 5’  | 64.7          | 4.04, m, 2                              | 0.34              |                   |
| 1st | 27.7          | 1.89, m, 7b                             | 23.5, 21.6        |                   |
| 2st | 34.9          | 3.45, m, 4                              | 23.5, 21.6        |                   |
| 3st | 172           |                                        |                   |                   |
| 4th | 21.7          | 2.04, s, 5                              | 172.1, 173.9      |                   |

aThe preparation also contained 2NAcAmEtPn and small amounts of one or more contaminants. 13C and 1H NMR spectra were assigned to correlations observed in the COSY, HSQC and HMBC NMR spectra. 1H/13C and 1H/31P HMBC NMR correlations are shown.

Discussion

Acetylation of aminoalkylphosphonates is an efficient process in E. coli. A culture of a phn+ strain, such as HO2569, which expressed the phn operon constitutively, removed essentially all of the added 2AmEtPn (2 mM) in approximately 24 h. Thus, acetylation of 2AmEtPn by far exceeds the phosphate-need, which is less than 0.4 mM. The efficiency of the process may be related to the detoxification-effect. Acetylation of S1AmEtPn, i.e. detoxification, must be efficient to prevent cell-lysis. It is possible, that phosphonate is taken up at a rate higher than that of the catabolism in order to keep the catabolic machinery saturated. With S1AmEtPn, non-acetylated compound would be detrimental to the cell and to prevent this, S1AmEtPn is efficiently acetylated to SNAc1AmEtPn and some of this compound is catabolized, whereas the surplus is released to the growth medium. This overwhelming quantitative acetylation-process, furthermore, suggests that acetylation precedes C–P bond cleavage. The fact that this, indeed, is the case was confirmed by the isolation of a substrate alaphosphin (L-alanyl-L-1-aminoethylphosphonate). Acetylation of aminoalkylphosphonates was catalyzed by phnO-specified aminoalkylphosphonate-N-acetyltransferase. Thus, the phnO-deficient strains HO2541 and HO3412 were unable to acetylate 2AmEtPn. Despite of this lack of acetylation, 2AmEtPn was an excellent phosphate source for the phnO-deficient strains. Furthermore, AmMePn and S1AmEtPn were readily acetylated in vivo, and served as P source for phnO+ strains in vivo, but not for phnO strains. In fact, the most prominent physiological difference between a phn+ and the phnO strains was the inability of the phnO strains to remove aminoalkylphosphonate from the growth medium, and their lack of growth on 1-aminoalkylphosphonates (i.e. AmMePn or S1AmEtPn) as P source.

It is likely that one or more enzymatic steps of the C–P lyase pathway are hampered by the presence of a free amino group close to the phosphorus atom of the phosphonate group. One possibility for the catabolic stability of AmMePn is the effect of the amino group on the stability of the carbon-centered radical upon C–P bond cleavage. At physiological pH the amino group of AmMePn can be expected to be protonated and have a formal positive charge. It is estimated that the electron withdrawing effect of an ammonium group destabilizes an attached carbon centered radical by 4–5 kcal mol⁻¹ [15,16]. This has been used to explain the regioselectivity of radical formation at carbon atoms remote from the α-amino group of amino acids or the α-amino group of lysine [17]. Conversely, N-acetylation would neutralize this charge, as well as assist radical formation by delocalization of the single electron into the acetyl group. The inductive effect of the ammonium group may also explain why 2AmEtPn can undergo C–P bond cleavage without acetylation by aminoalkylphosphonate-N-acetyltransferase, since this effect will be weaker when the electron-withdrawing group is placed further away from the site of radical formation. Acetylation serves an additional role in detoxification. S1AmEtPn, an analog of γ-alanine, causes inhibition of alanine racemase and inhibition of peptidoglycan biosynthesis resulting in cell lysis. S1AmEtPn can be supplied to E. coli as the suicide compound alaphosphin (L-alanyl-L-1-aminoethylphosphonate). After uptake of alaphosphin by a dipeptide transport system followed by hydrolysis by a dipeptidase S1AmEtPn is formed and exerts its bacteriocidal effect [14]. Our results confirmed the toxic effect of S1AmEtPn on cell wall biosynthesis after addition of the free compound. In a phn+ strain the bacteriocidal effect of S1AmEtPn could be overcome by the addition of P, or γ-alanine or both. However, phnO strains were much more susceptible to inhibition than phn+ strains. Thus, even with P, or γ-alanine present, S1AmEtPn was toxic for the phnO strains. Specifically, the fact that the phn+ strain grew in the presence of S1AmEtPn and P, whereas the phnO strains did not, demonstrates that the acetylation mediated by aminoalkylphosphonate-N-acetyltransferase is a requisite for detoxification of S1AmEtPn. Under these conditions S1AmEtPn need not function as P source. Additionally, the fact that the phn+ strain grew in the presence of S1AmEtPn and γ-alanine, whereas the phnO strains did not, demonstrates that the
acetylation is a requisite for utilization of S1AmEtPn as Pi source. Although C-P lyase is widely spread among bacterial species, many phn operons lack a phnO citron. An example of these microorganisms is Pseudomonas stutzeri. This organism contains two C-P lyase specifying operons (htx and phn) none of which contain a phnO homolog [10]. Whereas 2AmEtPn is an excellent source of Pi, the growth response of P. stutzeri to AmMePn or S1AmEtPn has not been reported, but if the C-P lyase pathway(s) of this organism share properties with that of E. coli, we predict that P. stutzeri is unable to utilize neither AmMePn nor S1AmEtPn. Alternatively, a phnO homolog may be located outside the htx and phn operons or some unspecific acetyltransferase may participate in detoxification and catabolism of AmMePn and S1AmEtPn in P. stutzeri. Furthermore, in C-P lyase-less S. enterica aminoalkylphosphonate N-acetyltransferase may serve as a detoxifying enzyme as previously suggested [9].

Although our data demonstrates a physiological role of aminoalkylphosphonate N-acetyltransferase, acetylation of AmMePn and S1AmEtPn, it remains to be established if AmMePn and S1AmEtPn are naturally produced compounds. Additionally, it is possible that other 1-aminoalkylphosphonates are also substrates for aminoalkylphosphonate N-acetyltransferase and that some of these compounds are naturally produced. Indeed, AmMePn is a prominent intermediate in the catabolism of the man-made herbicide glyphosate in soil [19].

By $^{32}$P-labeling of catabolic intermediates we previously showed that phnO strains of E. coli accumulate at least two radioabeled compounds when fed alkylphosphonate or aminoalkylphosphonate. Both compounds contained a radioabeled phosphate ester and we previously showed that one of these compounds is 5-phospho-$\alpha$-$\delta$-ribose 1,2-cyclic phosphate [7,12,20]. Due to the behavior in TLC we concluded that the second compound additionally contained a phosphonyl moiety. We show here that this second compound is 5'-phospho-$\alpha$-$\delta$-ribose 1'-phosphate. In the case of 2AmEtPn-grown cells the accumulated compound is 5'PRib1'2NAcAmEtPn. We previously postulated that 5'-phospho-$\alpha$-$\delta$-ribose 1'-phosphate is the substrate of C-P lyase. This was subsequently shown by in vitro analysis to be the case [5–7]. Thus, the detection of 5'PRib1'2NAcAmEtPn in the culture medium of E. coli is the first demonstration in vivo of a substrate for C-P lyase. The fact that 5'PRib1'2NAcAmEtPn carries an acetyl group definitively proves that acetylation of aminoalkylphosphonate precedes C-P bond cleavage by C-P lyase.

The facts that phnO strains efficiently utilize 2AmEtPn as Pi source and that phnO+ strains efficiently acetylate 2AmEtPn raises the question whether there is simultaneous utilization of 2AmEtPn, acetylated and non-acetylated compounds. Our data indicate that in phn wild-type strains there is one dominant pathway. Thus, in the low-field region of $^{31}$P NMR spectroscopy only a single C-P containing compound was observed (δ 23.6 ppm, Table 3). This chemical shift is consistent with Ribi1'2NAcAmEtPn, i.e. 2AmEtPn acetylated and attached to ribose. Had there been also a non-acetylated derivative present (i.e. $\alpha$-$\delta$-ribose 1'-$N$-(2-aminoethyolphosphonomo)), two peaks had been expected in this region of the $^{31}$P NMR spectrum. However, the concentration of such an intermediate, signifying a second pathway, may be below the detection limit of $^{31}$P NMR spectroscopy.

Figure 1 shows the conversion of phosphonate to phosphate ion. Although the structure of most of the intermediates have now been discovered, the terminal steps of the pathway have not firmly been set. It has been suggested that PRPP is the product of the phn-specified reactions. PRPP is the product of phnN specified ribosyl 1,5-phosphosphate phosphokinase activity. The processing of PRPP may involve the activity of one or more phosphoribosyltransferases (PRTases), which produce PP, followed by the activity of inorganic diphosphatase, which completes the formation of P.[6].This pathway is attractive, as organisms such as E. coli contain 10 PRTases. PRPP is an important intermediate of purine, pyrimidine and pyridine nucleotide biosynthesis as well as histidine and tryptophan biosynthesis. At least purine and pyrimidine PRTases are generally constitutively synthesized, and, thus, present at all times and available for diphosphorylation of PRPP formed also by phosphate degradation [21]. In addition, inorganic diphosphatase, specified by ppa, is essential for E. coli, and, similarly to PRTases, present at all times [22].

Although we did not perform a detailed kinetic analysis of E. coli aminoalkylphosphonate N-acetyltransferase, we noticed at least one difference from the S. enterica aminoalkylphosphonate N-acetyltransferase. The E. coli enzyme was able to acetylate R1AmEtPn, which is not a substrate for the S. enterica enzyme [9]. Kinetic analysis of the S. enterica enzyme revealed that S1AmEtPn was the most efficient substrate ($k_{cat}/k_{M}$ value 7.8 x $10^4$ M$^{-1}$ s$^{-1}$) compared to 4.1 x $10^3$ and 5.0 x $10^3$ M$^{-1}$ s$^{-1}$ for AmMePn and 2AmEtPn, respectively. Additionally, the k$\text{cat}$/k$M$ values diminished for 1-aminoalkylphosphonates with longer alkyl chains [9]. Thus, the high k$\text{cat}$/k$M$ value for S1AmEtPn is consistent with a dual function of acetylation in detoxification and catabolism. Perhaps the acetylation of aminoalkylphosphonates other than AmMePn and S1AmEtPn is without a physiological function, but has evolved as a redundant side effect.

Finally, our results may be applicable to the degradation mechanism of environmental AmMePn, also called AMPA. AMPA/AmMePn is an important metabolite in the catabolism of glyphosate ($N$-methylphosphono)glycine), the active compound of the herbicide Roundup. Glyphosate can be degraded by either of two pathways both of which involve C-P lyase. In one pathway C-P lyase cleaves glyphosate to Pi, and N-methylglycine (sarcosine), which is further degraded in intermediary metabolism. In the other pathway glyphosate is cleaved to AmMePn and glyoxylate, which is catalyzed through the glyoxylate cycle [23]. AmMePn very likely is converted to Pi, and N-methylacetamide by the C-P lyase pathway with the inclusion of the phnO gene product as described in Figure 1. In some organisms N-methylacetamide may be further degraded as it has been reported that AmMePn is catalyzed to CO$_2$ [24]. Alternatively, glyphosate-catabolism may include a different acetylation step. Thus, an enzyme with glyphosate N-acetylation activity has been discovered in Bacillus licheniformis. The physiological importance of this enzyme remains to be elucidated [25].

Materials and Methods

General

Organophosphonates, d-alanine, d,L-alanine and acetylcoenzyme A were obtained from Sigma-Aldrich. NMR spectra were recorded on Bruker Avance 400, 500 or 600 MHz spectrometers. $^1$H NMR chemical shifts (δ) are reported relative to HDO, whereas $^{31}$P NMR chemical shifts are reported relative to 17 mM phosphoric acid as an external standard. ESI-MS analysis was purchased at Queen’s University Mass Spectrometry and Proteomics Unit and was performed with an Applied Biosystems/MDS Sciex QStar XL MS instrument.

Bacterial strains and growth conditions

The E. coli K-12 strains used as well as their construction are shown in Table 5. In strain HO2735 the lacP-specified repressor served to repress transcription of genes harbored in the pUHE23-2
vector. \textit{phn}E(\textit{EcoB}) indicates that the \textit{phn} operon originates from wild-type \textit{E. coli} K-12, which is phosphonate growth-deficient, due to an 8-bp duplication in the \textit{phn}E cistron. \textit{E. coli} K-12 strains can be made phosphonate growth-proficient by selection for growth with phosphonate as sole Pi source. These phosphonate growth-proficient mutants have lost the 8-bp duplication and are designated \textit{phn}\textit{E} \textit{EcoK}' [26,27]. Liquid growth medium was Tris-buffered 0.3P minimal medium containing 0.3 mM Pi, or Pi-free Mops-buffered minimal medium [12,28]. Glucose (0.2%) was used as carbon source. Organophosphonates were used at concentrations of 0.3 or 2.0 mM, l- and d-alanine were used at a concentration of 100 mg L\textsuperscript{-1}. To analyze conversion of phosphonate, cells were grown in 0.3P medium to an optical density at 600 nm (OD\textsubscript{600}) of 0.45, at which time phosphonate was added to a concentration of 2 mM. Samples (3 or 5 mL) were removed at time intervals, or alternatively after 20 h of incubation, and centrifuged to remove cells. The supernatant fluid was analyzed immediately or stored at -20°C. Solid medium was Mops-buffered minimal medium containing 1.8% agar. Glassware and agar were washed with deionized water (Milli-Q system) to reduce undesired Pi-content.

Purification and characterization by NMR of 2NAcAmEtPn

A culture of strain HO2542 (\textit{phnP}) [250 mL of 0.3P medium] was grown to an OD\textsubscript{600} of 0.5 at which time 2AmEtPn was added to 2 mM and incubation continued for 24 h. Following centrifugation the supernatant fluid was loaded on a formate form of an AG1-4X column (2.5 x 30 cm). After wash with 300 mL of deionized water, a gradient of 0-4.0 M ammonium formate in 0.1 M formic acid was used for elution. The flow rate was 0.5 mL min\textsuperscript{-1}. \textsuperscript{31}P NMR was used to analyze fractions for phosphorus-containing compounds. Quantification was achieved by the inclusion of an external standard of 17 mM phosphoric acid. The elution profile is shown in Figure S2A. Fractions showing \textsuperscript{31}P NMR chemical shift 18.4 ppm (i.e. elution volume 97 to 115 mL) were pooled and the solvent removed repeatedly in vacuo to afford 2NAcAmEtPn.

Purification and characterization by NMR of 5'PRib\textsubscript{1}2NAcAmEtPn

The two fractions representing elution volume 125 to 130 (6 mL) (Figure S2B) were combined, lyophilized repeatedly in vacuo to afford 5'PRib\textsubscript{1}2NAcAmEtPn. As the elution profile was established by \textsuperscript{31}P NMR of each fraction, the final fraction of 5'PRib\textsubscript{1}2NAcAmEtPn contained one or more additional compound(s) without phosphorus atoms as contaminants.

DNA methodology

A \textit{phn} \textit{O} variant specifying aminooalkylphosphonate \textit{N}-acetyltransferase with a six-histidine tail at the carboxy terminus (\textit{phnO}_{\text{His\textsubscript{6}Tail}}) was prepared by PCR with the four deoxyribonucleoside triphosphates, the oligodeoxyribonucleotides 5'-GAAGLATT-CATTAAAGAGGAAATTTAATCATGCGCTCCTTGAG CTTGCGCGGGCCACGC-3' and 5'-TGGCCAATGGTTAT-

\textit{TAaagggaggagggAGCGCCCTGGGAAACGGGAGAGTG GCTGTCGTGTCGTGGCTCCGGC-3' as primers (nucleotides specifying translation initiation and stop codons are shown in bold and nucleotides specifying a hexahistidine-tail are shown in lower case), DNA of strain HO1429 as the template and Vent DNA polymerase (New England Biolabs). The resulting DNA fragment was restricted by EcoRI and Nof (recognition sites are shown in italics in the sequences above) and the liberated 479-bp DNA

Table 5. \textit{E. coli} strains used.

| Strain           | Relevant genotype                                                                 | Reference or construction |
|------------------|-----------------------------------------------------------------------------------|----------------------------|
| BW14001          | *\textit{mel-pap*}-\textit{phnCDEFGHKLMNPO}2:TN5seq1/132(tet)                   | [2]                        |
| BW14894          | *\textit{phnCDEFGHKLMNPO}23-30                                                  | [29]                       |
| BW17572          | \textit{phnP} [\textit{EcoB} \textit{phnO38:TrphO}A-9]                         | [6]                        |
| BW25113\textsuperscript{a} | \textit{phnP} [\textit{EcoK}']                                | [30]                       |
| HO1429           | \textit{phnP} [\textit{EcoK}']                                        | [7]                        |
| HO2536           | \textit{phnP} [\textit{EcoB} \textit{phnO38:TrphO}A-9 \textit{pstS605:cat}]  | [31]                       |
| HO2541           | \textit{phnP} [\textit{EcoB} \textit{phnO38:TrphO}A-9 \textit{pstS605:cat}]  | [6]                        |
| HO2542           | \textit{phnP} [\textit{EcoB} \textit{phnP54:TrphO}A-1 \textit{pstS605:cat}]  | [6]                        |
| HO2568           | \textit{phnP} [\textit{EcoB} \textit{pstS605:cat}]                             | [31]                       |
| HO2678           | *\textit{phnPCTDEFGHKLMNPO}23-30                                              | P1(BW14894) x BW14001 Mel\textsuperscript{b} |
| HO2780           | *\textit{phnPCTDEFGHKLMNPO}23-30                                              | P1(BW14001) Mel\textsuperscript{b} |
| HO2735           | *\textit{phnPCTDEFGHKLMNPO}33-30                                              | [31]                       |
| HO3412           | *\textit{pstS605:cat phnP} [\textit{EcoK}'] or \textit{phnP} [\textit{EcoB} \textit{phnO789:kan}] | P1(UW4054-4) x HO2568, Kan\textsuperscript{b} |
| HO3413           | \textit{phnP} [\textit{EcoK}'] \textit{phnO789:kan}                            | JW4054-4, MePn as P, source [7] |
| HO3414           | \textit{phnP} [\textit{EcoK}'] \textit{phnO789:kan}                            | BW25113, MePn as P, source [7] |
| HO3417           | \textit{phnP} [\textit{EcoK}'] \textit{pstS605:cat}                           | P1(HO2568) x HO3414, Cmlr\textsuperscript{b} |
| HO3418           | \textit{phnP} [\textit{EcoK}'] or \textit{phnP} [\textit{EcoB} \textit{phnO38:TrphO}A-9] | P1(BW17572) x HO3414, Kan\textsuperscript{b} |
| JW4054-4\textsuperscript{a} | \textit{phnP} [\textit{EcoK}'] \textit{phnO789:kan} | [30]                       |

\textsuperscript{a}Purchased from the Coli Genetic Stock Center, Yale University, New Haven, CT.

\textsuperscript{b}Bacteriophage P1-mediated transduction [33]. Selection was for growth with melibiose as carbon source (Mel\textsuperscript{+}), kanamycin resistance (Kan\textsuperscript{+}) or chloramphenicol resistance (Cmlr).

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fragment was ligated to similarly restricted DNA of pUHE23-2 (provided by H. Bujard, University of Heidelberg, Germany). The insert of the resulting plasmid, pHO512, was sequenced and found to have the expected nucleotide sequence including the six histidine-specifying codons.

Purification and assay of aminoalkylphosphonate N-acetyltransferase
To purify phnO-specified aminoalkylphosphonate N-acetyltransferase, strain HO2735 (Ap::F lacP zze::Tn10, pHO512 (phnO_C6xHis)) was grown in LB (0.5 L) at 37°C with aeration by shaking until an OD₆₀₀ of 0.7 was reached, at which time the culture was cooled in ice for 30 min and 36 mg of isopropyl β-D-1-thiogalactoside was added to induce phnO gene expression. Incubation then continued with shaking at 27°C for 6 hours. Cells were harvested by centrifugation, resuspended in 25 mM sodium phosphate buffer, 0.3 M sodium chloride, 0.25 M imidazole, pH 8.0, and homogenized in an Emulsiflex (model C5, Avestin, Ottawa, ON). Debris was removed by centrifugation, and the supernatant fluid was loaded on a 2-mL column of Ni-NTA agarose (Qiagen, Hilden, Germany). After wash with 25 mM sodium phosphate buffer, 0.3 M sodium chloride, 20 mM imidazole, pH 8.0, protein was eluted with repeated additions of 0.5 mL of 25 mM sodium phosphate buffer, 0.3 M sodium chloride, 0.25 M imidazole, pH 8.0. Elution of aminoalkylphosphonate N-acetyltransferase was followed by SDS-PAGE. Fractions 7 to 10 were pooled and dialyzed against 25 mM sodium phosphate buffer, pH 8.0. The purity of aminoalkylphosphonate N-acetyltransferase was assessed as more than 95% as evaluated by SDS-PAGE.

To assay the activity of aminoalkylphosphonate N-acetyltransferase, purified enzyme (10 μL) was added to a reaction cocktail (final volume of 0.6 mL) with final concentrations of aminoalkylphosphonate, acetylcoenzyme A and magnesium chloride of 1, 3, and 3 mM, respectively and 25 mM Tris/HCl cocktail (final volume of 0.6 mL) with final concentrations of 0.3 M sodium chloride, 0.25 M imidazole, pH 8.0. Elution of aminoalkylphosphonate N-acetyltransferase was followed by SDS-PAGE. Fractions 7 to 10 were pooled and dialyzed against 25 mM sodium phosphate buffer, pH 8.0. The purity of aminoalkylphosphonate N-acetyltransferase was assessed as more than 95% as evaluated by SDS-PAGE.

To assay the activity of aminoalkylphosphonate N-acetyltransferase, purified enzyme (10 μL) was added to a reaction cocktail (final volume of 0.6 mL) with final concentrations of aminoalkylphosphonate, acetylcoenzyme A and magnesium chloride of 1, 3, and 3 mM, respectively and 25 mM Tris/HCl buffer, pH 8.0. The disappearance of aminoalkylphosphonate and the appearance of acetylated product were followed by 31P NMR.

Supporting Information
Figure S1 Growth response of strain HO2568 (phnO_AptsS) to S1AmEtPn. The growth medium was 0.3% Squares show growth with L-Alanine present, circles show growth without D,L-alanine. S1AmEtPn was added at time zero. Growth was followed as described in Materials and Methods. (TIF)

Figure S2 Elution by ion-exchange chromatography of phosphorus containing compounds generated by strain HO2542 (phnP) after 24 h of incubation at 37°C in the presence of 2NAcEtPn. The used growth medium was added to the column and elution was analyzed by 31P NMR spectroscopy. The relative amounts of the various compounds were estimated with an external standard consisting of 17 mM phosphoric acid. Black squares correspond to phosphate compounds with a chemical shift of δ 24 ppm (Rib1’2NAcAmEtPn for elution at 50 to 97 mL, 5’Rib1’2NAcAmEtPn for elution at 116 to 138 mL); blue squares, δ 20 ppm (2NAcAmEtPn); purple circles, δ 18.6 ppm (Rib1,2cP); red circles, δ 3.6 ppm (5’-phosphate of 5’Rib1’2NAcAmEtPn); green circles, δ 1.6 ppm (Pi). (A) Elution profile of all five compounds, (B) blow-up of the profile of the compounds with chemical shifts δ 24 (black squares) and δ 3.6 ppm (red circles). (TIF)

Figure S3 Time course of conversion of AmMePn by strain HO2542 (phnP). Squares, AmMePn (δ 9.2 ppm); diamonds, NAcAmMePn (δ 13.9 ppm); triangles, Rib1’NAcAmMePn (δ 17.4 ppm); circles, Rib1,2cP (δ 18.6 ppm). (TIF)

Figure S4 Time course of conversion of AmMePn by strain HO2536 (phnP). Squares, AmMePn (δ 9.2 ppm); diamonds, NAcAmMePn (δ 13.9 ppm); triangles, Rib1’NAcAmMePn (δ 17.4 ppm). (TIF)

Figure S5 1H/1H COSY spectrum of 5’PRib1’2NAcAmEtPn. Protons of 2NAcAmEtPn are labeled in red. (TIF)

Figure S6 1H/13C HMBC spectrum of 5’PRib1’2NAcAmEtPn. Carbons and protons of 2NAcAmEtPn are labeled in red. (TIF)

Author Contributions
Conceived and designed the experiments: BHJ. Performed the experiments: BHJ FRM. Analyzed the data: BHJ FRM DLZ. Wrote the paper: BHJ FRM DLZ.
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