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The ABC of phosphonate breakdown: A mechanism for bacterial survival

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Abbreviations: C-P lyase, carbon-phosphorus lyase; ABC, ATP-binding cassette; SAM, S-adenosyl methionine

ABSTRACT

Bacteria have evolved advanced strategies for surviving during nutritional stress, including expression of specialised enzyme systems that allow them to grow on unusual nutrient sources. Inorganic phosphate (P_i) is limiting in most ecosystems, hence organisms have developed a sophisticated, enzymatic machinery known as carbon-phosphorus (C-P) lyase, allowing them to extract phosphate from a wide range of phosphonate compounds. These are characterised by a stable covalent bond between carbon and phosphorus making them very hard to break down. Despite the challenges involved in both synthesizing and catabolising phosphonates, they are widespread in nature. The enzymes required for the bacterial C-P lyase pathway have been identified and for the most part structurally characterised. Nevertheless, the mechanistic principles governing breakdown of phosphonate compounds remain enigmatic. In this review, we provide an overview of the C-P lyase pathway and discuss structural aspects of the involved enzyme complexes with a special emphasis on the role of ATP-binding cassette (ABC) proteins.

1.Introduction

Bacteria are uniquely adaptable organisms, able to survive under highly challenging conditions including drought, heat, and nutritional starvation. Starvation for nutrients is generally associated with the so-called "stringent response", which is a wide-ranging adaptation of metabolism to sustain life during the times of hardship.^[1] During the stringent response, the related cellular alarmones, 5'-diphosphate-guanosine-3'-diphosphate (ppGpp) and 5'-triphosphate-guanosine-3'-diphosphate (pppGpp), are produced in high quantities and thought to be responsible for orchestrating a global reprogramming of metabolism, including inducing a complete shutdown of transcription, translation, and cell division.^[1]

Starvation for phosphorus (P_i) is particularly challenging as this macronutrient is much less abundant in the biosphere than carbon, nitrogen, oxygen, and hydrogen, while it is still required in relatively large quantities during growth for incorporation into nucleic acids, phospholipids, enzyme co-factors, and for energy conversion. Most organisms take up phosphorus in the form of phosphate, which is highly soluble and can be readily handled enzymatically due to the reactivity of phosphate esters. But phosphate is scarce and limiting for growth in most natural ecosystems, so some organisms have developed alternative ways of incorporating more reduced forms of phosphorus in their metabolism.^[2] Phosphonate compounds, organic compounds that contain a direct covalent bond between adjacent carbon and phosphorus atoms (the C-P bond), represent one such source. Small molecule phosphonate compounds, such as methylphosphonate ($H_3C-PO_3^{2-}$, Figure 1A), which is the simplest phosphonate and 2-aminoethylphosphonate, are ubiquitous in nature, but phosphonates are also found as constituents of lipids, polysaccharides, and polypeptides (Figure 1A).^[3, 4] Phosphonolipids are particularly widespread, both in microorganisms and higher eukaryotes, and constitute up to 30% of membrane lipids in *Tetrahymena*.^[5] Since phosphonates are natural analogues of

phosphate esters and carboxylates, they are highly potent as inhibitors of enzymatic activities and therefore also form the basis for a range of natural antibiotics produced by several species of bacteria as secondary metabolites.[6] Equally important, from an environmental point of view, thousands of tons of manmade phosphonates are released to the environment each year as detergents and pesticides, including the world's most used herbicide, glyphosate (RoundUp™).[7]

Remarkably, a single enzyme is responsible for the formation of nearly all C-P bonds in nature, namely *phosphoenolpyruvate mutase*, which generates phosphonopyruvate from phosphoenolpyruvate and thus converts a regular phosphate ester into a phosphonate (Figure 1B).[8] Phosphonopyruvate then forms the starting point for biosynthesis of several other phosphonate compounds, including methyl- and ethylphosphonates in marine microbes[9] as well as natural antibiotics, including the potential antimalarial agents FR-900098, phosphonoalanine, and valinophos.[3, 10] Phosphonopyruvate may be decarboxylated to form phosphonoacetaldehyde, the immediate precursor of 2-aminoethylphosphonate, which again is a precursor of several other compounds with antibiotic effects.[10] Finally, reduction of the carbonyl of phosphonopyruvate results in the formation of 2-hydroxyethylphosphonate, a precursor of the natural antibiotics, phosphomycin, phosphinothricin tripeptide, and dehydrophos.[3, 11]

At the other end of the spectrum, a wide range of bacterial species are able to convert phosphonates into phosphate for growth by one of three mechanisms: Radical-based cleavage, oxidative cleavage, or hydrolytic cleavage.[11-13] In general, the oxidative and hydrolytic cleavage mechanisms are associated with specific requirements of the substrate, such as the presence of a 2-carbonyl group in the vicinity of the C-P bond.[11] In contrast, radical-based breakdown by C-P lyase is the only process able to cleave unsubstituted phosphonate

compounds and release their corresponding hydrocarbon moieties. C-P lyase exhibits a remarkable lack of substrate specificity and is able to process an extremely wide range of phosphonate substrates, including both (amino-) alkyl (saturated as well as unsaturated) and aryl phosphonates as well as the H-P bond of phosphite.[11] Radical-based breakdown of phosphonate compounds was inferred in the 1980s by Frost and co-workers who noticed that rearrangement products were formed during cleavage of some phosphonates by C-P lyase.[14] They rationalised that this would only occur during a homolytic (i.e. radical) cleavage and not during a more traditional heterolytic or oxidative cleavage reaction. They also found that the process involved the formation of a *D*-ribosyl derivative.[15] In fact, radical-based breakdown of phosphonate compounds constitutes a pathway of five or six enzyme-catalysed reactions in many bacteria, *the C-P lyase pathway*. This pathway is one of only two ways that soil bacteria are reported to degrade the common herbicide, glyphosate.[12]

In this review, we focus on our current understanding of the C-P lyase pathway, which involves an exceptional multi-enzyme system able to cleave carbon-phosphorus bonds in the widest variety of phosphonate compounds.[16]

2. The C-P-Lyase pathway requires several multi-protein complexes

The C-P lyase pathway is initiated by activation of the phosphonate compound, followed by C-P bond cleavage and additional processing eventually leading to the formation of 5-phosphoribosyl 1-diphosphate (PRPP), a common intermediate in nucleotide, amino acid and co-factor metabolisms.[15, 17] In *E. coli*, the required enzymes and other proteins are all encoded by the 14-cistron *phnCDEFGHIJKLMNOP* operon[18], which is transcriptionally activated under phosphate-limiting growth conditions in order to enable the utilisation of phosphonates (Figure 2A and Table 1).[19] More specifically, the *phn* operon forms a part of the *pho* regulon, consisting of at least 30 genes in 9 transcriptional units that are expressed upon P_i starvation.[19]

While all 14 *phn* genes are required for growth on phosphonate by *E. coli*, *phnGHIJKLM* appear to constitute the minimal, catalytic cassette. Briefly, activation of the phosphonate compound occurs by displacement of the adenine moiety of ATP by the phosphonate. This ribosylation reaction is catalysed *in vitro* by PhnI, which bears similarity to the nucleoside hydrolases, but requires PhnG, PhnH and PhnL (Figure 2B, reaction 1).^[20] In the absence of the three other proteins, PhnI will simply displace adenine from ATP using water, and thus not transfer the phosphonate compound, even if this is present in the solution.^[20] The PhnI-dependent reaction is followed by hydrolysis of the α,β -phosphate anhydride and release of diphosphate by PhnM, resulting in the formation of 5-phosphoribosyl 1-phosphonate (Figure 2B, reaction 2). This reaction does not appear to be affected by other proteins of the pathway. 5-phosphoribosyl 1-phosphonate is then the substrate of the C-P lyase subunit PhnJ, which cleaves the C-P bond, liberates the carbon moiety of the original phosphonate compound and produces 5-phosphoribosyl 1,2-cyclic phosphate (Figure 2B, reaction 3). At this stage, the phosphonate phosphorus has been converted to a ribose-coupled cyclic phosphate ester, which can readily be hydrolysed by other enzymes. Two additional enzymes, PhnP and PhnN, are responsible for the formation of the final product, PRPP (Figure 2B, reactions 4 and 5), where the phosphorus atom originating from the phosphonate compound ends up as the 1'-phosphate group. The formation of P_i from PRPP requires two additional enzymes, not encoded in the *phn* operon, namely a phospho-ribosyltransferase, which removes the diphosphate moiety of PRPP (Figure 2B, reaction 6), and diphosphatase, which hydrolyses diphosphate to P_i (Figure 2B, reaction 7).^[12, 21] Apart from the enzymes directly involved in conversion of phosphonates, the *phn* operon also encodes an ABC transport system, which is responsible for cellular import of phosphonates, consisting of an ATP-binding cassette (ABC) protein (PhnC), a transmembrane protein (PhnE), and a periplasmic phosphonate-binding protein (PhnD).^[22] PhnD is of particular interest, as it likely determines the overall phosphonate specificity of the

pathway by gating entry of phosphonate compounds into the cell. In fact, the sequence of PhnD can be used to predict the phosphonate degradation capability of a given organism (reviewed in Horsmann *et al.*^[11]). Curiously, however, the *phn* operon encodes two other putative ABCs in addition to PhnC, namely PhnK and PhnL (Figure 2A, marked "ABC"). These proteins appear to be orphan ABCs based on their lack of an associated transmembrane/periplasmic transporter proteins and it is not currently known if they function as part of an ABC transporter complex or have other roles. Nevertheless, all three ABC proteins in the *phn* operon contain intact nucleotide-binding domains, including the Walker A, Walker B, ABC, D and Q loop motifs as well as the switch H loop (Figure 3).^[23] The *phn* operon appears to be unique among approximately 70 operons in *E. coli* encoding ABC transporter systems by having three different ABC nucleotide-binding domains but only one transmembrane protein.^[24] Based on genomic vicinity to *phnD* and *phnE*, *phnC* is presumed to encode the ABC module used by the transport system, whereas the precise functions of the proteins encoded by *phnK* and *phnL* are unknown. Intriguingly, PhnL was shown to be required for the PhnI catalysed step of the C-P lyase pathway *in vitro*^[20] while PhnK forms a complex with PhnGHIJ both *in vivo* and *in vitro*.^[25, 26] Both PhnK and PhnL are essential for phosphonate utilisation *in vivo* but it is not known which reaction requires PhnK (Figure 2A).^[18] In this context, it is interesting to note that proteins that physically associate into larger complexes tend to be expressed via translational coupling in bacteria, i.e. with the initiator codon of the downstream gene occurring prior to the stop codon of the preceding gene. For the *phn* operon, this would predict two sets of proteins that potentially might associate, namely PhnGHIJK and PhnLMNO, for which complex formation has been confirmed biochemically and structurally for PhnGHIJK (Figure 2A).^[25, 26]

3. Which reaction does the PhnGHIJ complex catalyse?

The most intriguing step of the C-P lyase pathway is the actual cleavage of the C-P bond by PhnJ, a putative member of the radical *S*-adenosyl methionine (SAM) enzyme family.^[27] Radical SAM enzymes are anaerobic enzymes containing a [4Fe-4S] cubane-like iron-sulphur cluster, which is coordinated to the protein by three cysteine residues.^[28] These residues are located in a conserved CX₃CX₂C motif, which is a hallmark of the radical SAM enzyme family.^[29] On the basis of four conserved cysteine residues in its C-terminus (Cys241, Cys244, Cys266, and Cys272) in a non-canonical CX₂CX₂₁CX₅C motif, *E. coli* PhnJ was proposed to be a radical SAM enzyme.^[27] Despite the motif being non-canonical, three of the residues (Cys241, Cys244, and Cys266) were shown to be able to support the iron-sulphur cluster necessary for catalysis, while Cys272 was shown to be required for catalysis and likely carry a thiyl radical during part of the reaction.^[27]

Common to all radical SAM enzymes is that they are activated by reduction of the [4Fe-4S] cluster from the inactive +2 to the active +1 oxidation states by cellular electron carriers such as flavodoxin.^[28, 30] The reduced cluster is then believed to transfer an electron to a bound molecule of *S*-adenosyl methionine resulting in reductive cleavage of the co-factor. The resulting 5'-deoxyadenosyl radical can then abstract a hydrogen atom and generate a radical on a substrate, or in the case of glycy radical enzymes, directly on the backbone of a conserved glycine residue in the enzyme itself. Based on careful deuterium exchange experiments, PhnJ has been proposed to use a glycy radical species in catalysis, although it is not structurally similar to known glycy radical enzymes.^[27] These experiments also uniquely identified the universally conserved Gly32 as the location of the glycy radical. Furthermore, it was proposed that the radical would be transferred to Cys272 during the reaction, which then would attack and cleave the C-P bond on the substrate and return the radical to Gly32 and thus regenerate the enzyme.^[27]

The first structure of a component of the C-P lyase pathway was that of *E. coli* PhnH, which revealed a compact fold related to the pyridoxal 5'-phosphate-dependent transferases and showed that the protein forms a homodimer (Figure 4A).^[31] The significance of this observation was not understood until the crystal structure of the larger C-P lyase core complex (PhnGHIIJ) was determined, which revealed that PhnJ and PhnH have similar folds and interact tightly in a heterodimer very similar to the isolated PhnH homodimer (Figure 4B).^[26] This observation was surprising because earlier cross-linking studies had failed to show any interaction between PhnH and PhnJ.^[32] Overall, the C-P lyase core complex is a two-fold symmetrical dimer of PhnGHIIJ heterotetramers (Figure 4C). Although a significant number of the proteins required for phosphonate breakdown are present in this large structure, it is somewhat enigmatic and probably poses more questions than it answers. First of all, in the light of the requirement for the individual Phn proteins in the overall reaction, it is uncertain what catalytic competence the structure represents. The first reaction of the pathway, in which the phosphonate compound reacts with ATP, is catalysed by PhnI but also depends on the presence of PhnG, PhnH, and PhnL, of which PhnL is not present in the core complex. Given that PhnL is an ABC cassette protein, however, it is tempting to speculate that it might supply ATP to the reaction (Figure 2B). In the second step of the proposed reaction pathway, PhnM cleaves a diphosphate moiety from the ribosyl-1-phosphonate resulting from step 1, but PhnM is also not part of the core complex, which could suggest that the intermediate has to leave the core complex before returning for step 3, in which PhnJ, a central part of the core complex, cleaves the C-P bond (Figure 2B). In other words, the only step, which given our current knowledge can be catalysed by the isolated PhnGHIIJ complex, is step 3 which only depends on PhnJ. Surprisingly, this step has been shown to proceed in the absence of the other proteins *in vitro*.^[27]

4. How does the radical-based reaction catalysed by PhnJ proceed?

Another highly challenging aspect in terms of understanding catalysis by the core complex relates to the relative locations of Gly32, Cys272, and the [4Fe-4S] cluster within PhnJ. We know from other structures of radical SAM enzymes that the co-factor must bind directly adjacent to the [4Fe-4S] cluster during cleavage and radical generation (reviewed in Frey *et al.*^[33]). Moreover, the transfer of a hydrogen atom between Gly32 and Cys272 would suggest that these residues should also be located in the vicinity of the cluster. However, the structure of the C-P lyase core complex clearly demonstrated that while Cys272 is located right next to the cluster site (as defined by the other three cysteine residues, Cys241, Cys244, and Cys266), Gly32 is located more than 30 Å away on the other side of the protein close to the interface with PhnH (Figure 5A).^[26] Moreover, the catalytic Gly32 is buried and not exposed as would be expected for the required hydrogen exchange.^[33] Adding to this conundrum, careful deuterium exchange studies have demonstrated quite clearly, that 1) the pro-*R* hydrogen from one of the eight Gly residues in PhnJ ends up on the 5'-carbon of the 5'-deoxyadenosine (Ado-CH₃) cleavage product of SAM, and 2) that the pro-*S* hydrogen from one of these glycine residues is transferred to the final product, methane, resulting from cleavage of methylphosphonate by PhnJ (see Kamat *et al.*^[27] for a detailed reaction mechanism). The identity of the specific glycine residue involved was revealed partly through mass spectrometry and mutagenesis. Surprisingly, a Gly32Ala mutant is still active in cleaving SAM (it has a single backbone hydrogen) but cannot form the final product. This, together with the observation that when mutant (Gly32Ala) protein grown in the presence of deuterated alanine reacts with SAM, the deuterium ends up in Ado-CH₃, is only compatible with Gly32 being the location of the active site radical.^[27]

One caveat in interpreting the structure of the C-P lyase core complex relates to the fact that the protein complex was purified aerobically. Since the [4Fe-4S] cluster is not stable under aerobic conditions, a chelated zinc ion was found at the cluster site, similarly to what has been

observed for some other anaerobic iron-sulphur containing enzymes.[26, 34] It is therefore possible the core complex, under anaerobic conditions and in the presence of an intact iron-sulphur cluster, displays a different conformation that brings Gly32 closer to the cluster site. In this context, it is also worth noting that the X-shaped core complex contains a central hinge region, which in theory could rearrange and bring the "arms" of PhnH and PhnG closer to each other (Figure 4D). In support of this, a 7.8 Å cryo-EM structure of the C-P lyase core complex bound to PhnK (PhnGHIJK) showed that Gly32 becomes more exposed in the presence of PhnK, which may also promote radical transfer under the right conditions.[35]

5. Are there other active sites in the C-P-Lyase core complex?

Apart from C-P cleavage, the core complex is predicted to harbour one other activity, namely that represented by step 1 of the pathway, catalysed by PhnI. Structural analysis identified a putative location of the corresponding active site in a location where several histidine residues coordinate a zinc ion.[26] Histidine residues are common in active sites and a bound sulphate ion was furthermore found nearby that could possibly mimic a phosphate group on the incoming nucleotide (Figure 5B). Moreover, mutation of one or more of these residues prevents *E. coli* from growing on phosphonate as sole Pi source suggesting they are functionally critical[26]. Significant efforts have been put into determining a crystal structure of the core complex bound to substrates, intermediates, and products, but so far to no avail. It therefore remains unknown whether this site represents the "missing link" required for initiation of the C-P lyase pathway or is simply a structural zinc site. Following structure determination, it was also noted that there appears to be a cavity or tunnel going from the surface of the rather solid C-P lyase core complex to the histidine site.[26] It is therefore tempting to speculate that the phosphonate compound initially could enter the site along with ATP via this channel, and that the product of step, 1, 5-triphospho- α -D-ribosyl-1-phosphonate, likewise could exit in order to

be transferred to PhnM for step 2. Whether this would mean that PhnM physically docks on the C-P lyase core complex or remains in solution, is currently not known. However, it has not so far been possible to isolate a PhnM-containing complex after co-expression of PhnGHIJKLM which could indicate that PhnM is indeed a separate structural unit (BHJ, unpublished data).

6. What is the role of the ABC cassette protein PhnK?

A large number of the ABC modules found in Nature form part of ABC transporters and are as such associated with ATP-dependent transport of nutrients or other small molecules. However, there are several other groups of ABC modules that have different roles, including those in DNA repair and protein translation.^[23] For example, the *E. coli* excision nuclease, UvrA, contains three ABC modules and was the first ABC protein found to have a role unrelated to membrane transport.^[24, 36] Another *E. coli* ABC protein, EttA, has been identified as a member of ABCF subfamily and acts as a translation factor that gates ribosome entry into translation elongation cycle through a nucleotide-dependent interaction sensitive to ATP/ADP ratio.^[23, 37]

When co-expressed, the C-P lyase core complex (PhnGHIJ) forms a stable complex with a fifth protein, PhnK, which also shows homology to the ATP-binding cassettes (or nucleotide-binding domains, NBDs) found in ABC transporters.^[25] This larger, five-component PhnGHIJK complex can be purified to homogeneity, but it has not yet yielded crystals amenable to structure determination.^[26] Nevertheless, structural information is available from single-particle electron microscopy, initially from a 16 Å negative-stain model^[26], and later, from a 7.8 Å structure based on cryo-electron microscopy of native, unstained protein.^[35] These structures demonstrate quite convincingly that only a single molecule of PhnK, and consequently, a single ABC module, binds to the C-P lyase core complex thus breaking the

overall two-fold symmetry. It was shown both biochemically and structurally that PhnK interacts with a structural insertion segment in PhnJ, termed the *central insertion domain*, which is one of two expansion segments in the protein relative to the smaller, but structurally homologous PhnH. Consequently, it was also shown that removal of the central insertion domain prevents binding of PhnK to the core complex.^[26] This is consistent with cryo-EM, which revealed that a positively charged surface patch on PhnK interacts with a negatively charged region on PhnJ consisting of an α -helix and a loop.^[35] This observation is intriguing as the interaction thus appears to be "alphabetically appropriate" (PhnGHI**JK**), suggesting that the direct interaction between the two proteins is linked to their translational coupling.^[38] Moreover, the insertion domains of the two PhnJ molecules in the core complex are exposed in relatively close proximity on the same side of the particle, thus explaining why simultaneous binding of PhnK to both sites is not possible (Figure 6A). The functional implications of these stoichiometric relationships are not understood at present, but the observation suggests that the two otherwise identical halves of the core complex at some point during the catalytic process are no longer equivalent. One attractive possibility that, however, remains pure speculation, is that one half of the complex is responsible for step 1 of the reaction and the other half responsible for step 3. The observation that only a single PhnK subunit can bind to the core complex at any one time is also interesting in the light of the general function of ABCs, which usually form dimers with ATP located between the two cassette domains.^[39] Binding of PhnK has been shown to subtly alter the overall structure of the C-P lyase core complex so that the interaction between PhnJ and PhnH becomes looser resulting in exposure of the critical Gly32 residue.^[35] We can therefore also not exclude that a larger conformational change in the core complex, e.g. upon ATP binding, could shift the PhnK binding sites so much apart that another PhnK molecule could bind.

It is also tempting to speculate that the presence of an ABC transporter module on the surface of the C-P lyase core complex would allow this to localise to the inside of the plasma membrane with PhnK interacting with an ABC transporter transmembrane segment and perhaps catching incoming phosphonate compounds immediately as they emerge through the transporter. However, several lines of evidence suggest that this is not likely to be the case. First, as discussed above, a functional ABC transporter requires a dimer of ABC cassettes to effectively bind and hydrolyse ATP as well as open and close the transporter. Super-positioning of the PhnK-bound core complex onto known ABC transporter structures reveals that there would be a significant clash between core complexes if this were the case. In addition, the *phn* operon already encodes an ABC cassette subunit expected to form a functional transporter with the transmembrane protein PhnE, namely PhnC (Table 1). In other words, this subunit would have to be displaced in order for C-P lyase to bind to the transmembrane segment. Finally, the 7.8 Å cryo-EM structure of the C-P lyase core complex bound to PhnK established that the orientation of the PhnK subunit relative to the core complex is such that the helical region of PhnK (helices 3 and 4), which is responsible for connecting to the coupling helix of the transmembrane part in ABC transporters, is bound to the central insertion domain of PhnJ.^[35] In other words, it does not appear to be possible for PhnK to simultaneously bind a transmembrane segment and the core complex as the same binding interface is used in both cases (Figure 6B).

7. The *phn* operon encodes a third ABC cassette protein, PhnL

Intriguingly, the *phn* operon encodes yet a third ABC cassette protein adjacent to PhnK, namely PhnL. This protein is translationally coupled with PhnM, PhnN, and PhnO in *E. coli* (Figure 2A). As mentioned above, PhnL was shown to confer phosphonate specificity to step 1 of the pathway, where ATP is coupled to the phosphonate compound in a reaction catalysed by

PhnI.^[20] This experiment was, however, carried out with isolated components and PhnL was never shown to interact directly with the other three proteins required for this step, PhnG, PhnH, and PhnI. Moreover, since PhnK and PhnL are highly homologous, it might be speculated that PhnK, rather than PhnL, maintains this function *in vivo*. This would make sense from the point of view that PhnK is known to form a stable complex with the three other proteins, whereas PhnL is not. However, this would still leave open the question of the role of PhnL.

Figure 3 shows a sequence alignment of representative PhnK, PhnC, and PhnL sequences from a wide range of bacterial species identified using the NCBI Conserved Domain Database (CDD)^[40] along with two classical ABC cassette modules (MetN and MalK) from *E. coli* known to be involved in nutrient uptake via ABC transporters. This analysis allows us to identify both common features among the four groups of proteins as well as regions where they consistently differ. It is immediately evident by looking at the alignment that all groups (PhnK, PhnC, PhnL, and MetN/MalK) contain the complete set of canonical ABC cassette motifs, including the Walker A (P-loop), Q-loop, signature motif, Walker B, and H-switch motifs. This suggests that all groups of proteins are in fact functional in ATP binding and likely also ATP hydrolysis.^[39] But between the canonical motifs we can also identify sequence elements where the protein groups differ. Moreover, the well-characterised structure of ABC transporters as well as the EM reconstruction of PhnK bound to the C-P lyase core complex allow us to predict how these differences might affect binding and/or function of the individual proteins. The EM model predicts that the region between the Q-loop and the signature motif in PhnK (residues 90-145) is responsible for the interaction with the C-P lyase core complex (Figure 6C, purple). This region appears to be PhnK-specific in the sense that several stretches of residues seem to be conserved within the PhnK group but differ outside, e.g. ¹⁰¹SAG¹⁰³ and ¹¹⁴GARHYGDI¹²¹ (*E. coli* PhnK sequences, top line). This could suggest that these residues are directly involved

in binding the C-P lyase. Moreover, it suggests that neither PhnC nor PhnL would be able to bind in a similar fashion as they differ in these regions. As mentioned above, the same regions are also involved in the interaction between the ABC modules and the transmembrane segments in ABC transporters[39], such as the maltose transporter.[41] Surprisingly, none of the three Phn groups of ABC modules are very similar to the classical ABC proteins MetN and MalK, suggesting that this sort of analysis can be difficult.

Nevertheless, by studying the sequence gaps in the alignment, we can conclude that the PhnC group appears closest to MetN/MalK with respect to the region 60-70 and the gap around residue 80 (*E. coli* PhnK numbering), while PhnL is closer to the well-characterised ABC transporter subunits when it comes to the gap around 100-110. PhnK has a significant gap in this region, which is reminiscent of MetK/MalK, while PhnC has 6-8 residues inserted. When analysed in the context of the atomic model of PhnK bound to the C-P lyase core complex based on cryo-EM, this difference appears likely to extend a helix located in the PhnJ-binding region of PhnK by ~2 turns or alternatively extend the adjacent loop region (Figure 6C, green). Finally, the C-terminal third of the PhnK group (*E. coli* residues 175-225) also appear to include several stretches of residues that are specific to PhnK, such as the motif ¹⁸⁴LLDLLR¹⁸⁹ and ¹⁹⁹VVIV²⁰³ (Figure 3). In the binding model based on cryo-EM, these residues are located in the lower part of the ABC module, away from the C-P lyase core complex, suggesting that they may be relevant for interactions of PhnK with other proteins (Figure 6C, orange). In summary, while we cannot conclude based on sequence analysis which group of protein or proteins interact with the ABC transporter transmembrane segment, PhnE, we can identify unique sequence features among the PhnK, PhnC, and PhnL groups suggesting that only PhnK binds to the C-P lyase core complex at the binding site described by cryo-EM.

7. Conclusions

There is no doubt that the C-P lyase pathway still keeps a few secrets from us. Although we now know most of the individual protein structures, we still understand remarkably little about how catalysis proceeds for the first three, essential reactions of the pathway (Figure 2). The high-resolution structure of PhnGHIJ is not immediately compatible with the available functional and biochemical data suggesting that we are still missing either a major conformational state or one or more protein complexes of different composition. Of these, we know at least one, namely PhnGHIJK, however this has evaded high resolution structure determination so far. Confounding this, we also have no structures of substrate or product bound states of any of the enzyme subunits involved in the main C-P breaking reactions. This means that we do not know where the initial phosphonate substrate binds, if the PhnI product leaves the core complex to react with PhnM, or if the PhnM product re-enters to bind at the proposed second active site discovered at the interface between PhnI and PhnJ.^[26] Finally, we still know very little about the involvement of the three ABC proteins, PhnC, PhnK, and PhnL, in the pathway. The most likely scenario seems to be that PhnC is associated with the transmembrane transporter and that PhnK/PhnL bind ATP/ADP at some stage and either deliver one of these nucleotides to the reaction, or use the energy generated upon ATP hydrolysis for conformational changes required for the reaction. This, as well as answers to any of the other questions above, remains mere guesswork and speculation at this stage, and it is clear that we need additional structures of C-P lyase pathway components and their complexes, not the least in complex with relevant inhibitors, substrate analogues, stable intermediates, and products. Moreover, more careful functional studies, like those performed by Raushel and colleagues^[20, 27], to dissect the pathway and add biological value to such structures are essential as well. Only then, it seems, will the answers to the long-standing C-P lyase conundrum be given up by Nature.^[42]

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FIGURE LEGENDS

Figure. 1. Phosphonate compounds. **A.** Examples of phosphonate compounds found in Nature. Methylphosphonate is the smallest possible phosphonate compound, 2-aminoethylphosphonate is abundant in marine organisms, 1,2-diacyl-glycerol-3-(2-aminoethyl)-phosphonate is an example of a natural phosphonate lipid. **B.** A single enzymatic reaction catalysed by phosphoenolpyruvate mutase is responsible for almost all phosphonate synthesis in Nature.

Figure. 2. Overview of the C-P lyase operon and pathway. **A.** Overview of the *E. coli* *phn* operon with indication of known functions of the individual encoded proteins and reaction step in which each protein has been implicated. **B.** Pathway for the conversion of phosphonate to P_i . The phosphorus atom is shown in red and R- indicates a carbon moiety (or -H). In **1**, phosphonolysis replaces the adenine moiety of ATP with the phosphonate compound. The reaction is catalysed *in vitro* by PhnI, PhnG, PhnH and PhnL.^[20] The compound formed, 5-triphosphoribosyl 1-phosphonate, is the substrate for a triphosphoribosyl 1-phosphonate diphosphohydrolase (PhnM), which produces 5-phosphoribosyl 1-phosphonate and PP_i (**2**). 5-phosphoribosyl 1-phosphonate then is the substrate for C-P lyase (PhnJ) in **3**, which requires *S*-adenosylmethionine (*SAM*). The carbon moiety, indicated by R-H, is liberated, generating 5-phosphoribosyl 1,2-cyclic phosphate. In step **4**, a phosphoribosyl cyclic phosphodiesterase

(PhnP) hydrolyses the ester at C1 with the formation of *ribosyl 1,5-bisphosphate*. The C-P lyase pathway is terminated by the activity of *ribosyl bisphosphate phosphokinase*, which phosphorylates ribosyl 1,5-bisphosphate to PRPP (5). The following two enzymes responsible for the formation of P_i are not encoded in the *phn* operon. First, the activity of a *phosphoribosyltransferase* converts a nucleobase (e.g. adenine) and PRPP to a nucleoside 5'-monophosphate (NMP) (e.g. AMP) and PP_i (6) while a *diphosphatase* hydrolyses PP_i to P_i (7). The catabolism of 1-aminoalkylphosphonates furthermore requires the activity of the *N-acetyltransferase*, PhnO, which modifies the substrate before the phosphonolysis reaction (3).^[43]

Figure 3. Sequence alignment of bacterial PhnK, PhnC, and PhnL homologues. The sequences are grouped as PhnK (red), PhnC (blue), and PhnL (green) orthologues and were curated using the NCBI Conserved Domain Database (CDD).^[40] The sequence numbering at the top corresponds to the *E. coli* PhnK sequence (first sequence). The locations of the standard ABC motifs, i.e. the Walker A/P-loop, Q-loop, signature motif (Sign), Walker B, and H switch are indicated above the sequences. The region of PhnK proposed to be involved in binding of the core C-P lyase complexes (purple), extended helix region (green), and PhnK-specific regions (orange) are indicated as well. Below the PhnK/C/L sequences are shown two common ABC cassette proteins, MetN and MalK, involved in nutrient import of methionine and maltose via ABC transporters, respectively. Abbreviations: Eco, *Escherichia coli*; Pde, *Paracoccus denitrificans*; Rca, *Rhodobacter capsulatus*; Mlo, *Mesorhizobium loti*; Ret, *Rhizobium etli*; Sme, *Sinorhizobium meliloti*; Cvi, *Chromobacterium violaceum*; Bsp, *Burkholderia sp.*; Ace, *Acidithiobacillus ferrooxidans*; Ccr, *Caulobacter crescentus*; Bha, *Bacillus halodurans*; Sau, *Staphylococcus aureus*; Lla, *Lactococcus lactis*; Pae, *Pseudomonas aeruginosa*; Psy, *Pseudomonas syringae*; Bdi, *Bradyrhizobium diazoefficient*; Oih, *Oceanobacillus iheyensis*.

Figure 4. Structure of the C-P lyase core complex. **A.** Isolated *E. coli* PhnH (PDB 2FSU) forms a homodimer (green/light green).^[31] **B.** Inside the C-P lyase core complex, a similar interaction is found between PhnH (green) and PhnJ (red), which shares its core fold with PhnH.^[26] **C.** Overview of the C-P lyase core complex hetero-octamer. **D.** Side view of the core complex showing the two "arms" that protrude from a central joint possibly allowing structural flexibility.

Figure 5. Active sites in the C-P lyase core complex. **A.** Overview of the C-P lyase core complex with the two copies of PhnJ coloured red. The close-up shows the location of the two critical residues, Gly32 and Cys272, indicating the distance (~30 Å) between them. **B.** Another putative active site consisting of several His residues, a Zn²⁺ ion, and a sulphate ion, was located at the interface between PhnI and PhnJ.^[26]

Figure 6. Modelling of the interaction of PhnK with the C-P lyase core complex. **A.** Overview of the C-P lyase core complex with the two PhnJ molecules coloured light red. The CID region that has been shown to be responsible for binding PhnK is coloured dark red and a model for binding of PhnK based on the cryo-EM structure of PhnGHIJK is shown in yellow/purple/green cartoon next to a ghost surface representing binding of PhnK to CID on the other PhnJ molecule. The atomic model of PhnK is based the nucleotide-binding domain of a dipeptide ABC transporter (PDB 4FWI) and modelled using Phyre2.^[44] **B.** Structure of the *E. coli* vitamin B12 transporter BtuCD (PDB 4R9U)^[45] with one ATP-binding domain shown in the same orientation as PhnK in A and the PhnK model superimposed. **C.** Detailed view of the PhnK model bound to the C-P lyase core complex with relevant regions (PhnJ interaction, extended helix, and PhnK specific) coloured as in Figure 3.

Table 1. Biochemical function and structure of proteins encoded in the *E. coli phn* operon.

Protein	Activity	Structure*	References
PhnC	ABC transport system for phosphonate, nucleotide binding protein	N/A	[18]
PhnD	ABC transport system for phosphonate, periplasmic binding protein	3QK6	[46]
PhnE	ABC transport system for phosphonate, membrane-spanning protein	N/A	[47]
PhnF	Transcriptional regulator	2FA1	[48]
PhnG	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	4XB6	[26]
PhnH	Purine ribonucleoside triphosphate phosphonylase component, component PhnGHIJK	2FSU 4XB6	[26, 31]
PhnI	Purine ribonucleoside triphosphate phosphonylase component, component of PhnGHIJK	4XB6	[26, 49]
PhnJ	C–P lyase, component of PhnGHIJK	4XB6	[26]
PhnK	Component of PhnGHIJK, accessory protein, nucleotide binding protein of an ABC transport system.	EMD-6410	[26, 35, 38]
PhnL	Purine ribonucleoside triphosphate phosphonylase component or nucleotide-binding protein of an ABC transport system.	N/A	
PhnM	5-triphosphoribosyl 1-phosphonate diphosphohydrolase	N/A	
PhnN	Ribosyl bisphosphate phosphokinase	N/A	[50]
PhnO	Aminoalkylphosphonate <i>N</i> -acetyltransferase	N/A	[43, 51]
PhnP	Phosphoribosyl cyclic phosphodiesterase	3G1P, 3P2U	[18, 21, 52]

*Access codes refer to the Protein Data Bank (www.rcsb.org) or EM Data Bank (www.emdatabank.org)

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Figure 1

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Figure 2

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Figure 3

Figure 4

Figure 5

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Figure 6