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Factors Affecting the Expression of Phosphonate Metabolism in Gram-Negative Bacteria

By

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ABSTRACT

Phosphonates (Pns) are compounds that contain a direct C-P bond where P is in +3 oxidation state. Pn metabolism by microorganisms involves four different pathways but C-P lyase appears to be the most promising owing to its relatively broad substrate specificity. We studied phosphonate metabolism in two distant proteobacteria i.e. *E. coli* (γ-subdivision) and *Sinorhizobium meliloti* (α-subdivision).

E. coli can break down a restricted range of Pns via the C-P lyase pathway encoded by the *phn* gene cluster which is expressed under P starvation. However, *E. coli* K-12 is cryptic for utilisation of methylphosphonate (MePn) because of an 8bp insertion in the *phnE* gene potentially encoding the membrane component of an ABC-transporter. The octameric sequence is repeated directly 3 times in the *phnE* gene suggesting that slip strand mispairing may have caused the mutation. MePn utilisation in K-12 strains involves deletion of one of octameric repeats in *phnE.* Present studies revealed that octameric repeat deletion event is required for the growth of K-12 strains on other Pns i.e. ethyl phosphonate (EtPn), amino methyl phosphonate (AMPn), 2-amino ethyl phosphonate (2-AEP) and 3-amino propyl phosphonate (3-APPn).

We show that MePn-utilising variants of *E. coli* K-12 occur at high frequency ($\sim 10^{-2}$) in the populations not previously exposed to Pns. Populations no longer required to use MePn as sole P source i.e. stationary phase cultures switch back to the crypticity in Pn utilisation. Moreover when Phn^{+} variants of MC4100 obtained by selection to grow on MePn, EtPn, AMPn or 3-APPn reverted to crypticity (*phnE*$^{-}$ allele) at an equally high frequency when subcultured into LB. This shows that *phnE* gene in *E. coli* K-12 strains behaves as a high frequency ON⇔OFF phase switch similar to those observed in many other bacteria.

Although, four of the 11 independent newly isolated strains of *E. coli* were cryptic for growth on MePn, all contained double octameric repeats (*phnE*$^{+}$ allele) which is a characteristic of *E. coli* B strain. Moreover, *phnE* switch was inactive when cloned into the plasmid pTZ19R. Some replication, recombination and repair mutants that are known to enhance deletion of repeat units were tested for the behaviour of *phnE* switch. No evidence for the presence of ON form of *phnE* (two repeats) was found in the cultures of these strains growing on Pi. Deletion of one of the octameric repeats (OFF⇒ON switch) was observed in all cases when these strains were grown on MePn

as sole P source. In strains STL 1671 (*sbcB*), STL 2172 (*mutS*), STL2314 (*dnaQ49*^{TS}) and NR9807 (*dnaQ49*^{TS}) there was no reversion of *phnE* to the OFF form. However, ON⇒OFF switch (reinsertion of the octameric repeat) was observed in strain NR9458 that contains *mutD5* mutation which is an allele of *dnaQ*.

In *S. meliloti* 1021, C-P lyase pathway is induced in the presence of phosphonates and possesses broader substrate specificity as compared to that of *E. coli*. Structural genes (*phnGHIJK*) for the enzyme C-P lyase have been sequenced and characterized. A regulatory gene-like open reading frame oriented oppositely to *phnG* is present in *S. meliloti* 1021. This gene is similar to the *phnF* of *E. coli* which is a member of the GntR family of regulatory proteins.

A *phnF* deletion mutant of *S. meliloti* 1021 was constructed to evaluate the role of PhnF in transcription regulation of the *phn* operon. Using a promoter fusion to the reporter gene *lacZ*, we found that the *phnG* promoter activity was constitutive in the Δ*phnF* mutant strain both in the presence or absence of MePn which indicates that PhnF is a repressor of the *phn* operon. In the strain where *phnF* was supplied in trans on a plasmid, transcription activity was observed only when MePn was added to the culture medium indicating that MePn or a product resulting from MePn metabolism is the intracellular inducer of the *phn* operon. Although the Δ*phnF* mutant of *S. meliloti* was constitutive for *phnG* expression, surprisingly it did not grow in minimal medium containing MePn or low (10μM) inorganic phosphate as sole P source. Therefore, *phnF* may be required for the metabolism of phosphonates and also other P sources when present at low concentrations. One of the possible reasons could be that PhnF have a role in the regulation of phosphate and phosphonate transport genes.

The PhnF protein was overexpressed in *E. coli* as a thioredoxin fusion protein and purified to homogeneity. Its DNA binding activity and binding site were examined by gel retardation and footprinting assays and by site-directed mutagenesis of the binding sites *in vitro*. The PhnF showed binding to the target DNA regardless of the presence of MePn. PhnF binding site was found to be a 13bp pseudopalindromic sequence 5′-TATAAGATGTATA-3′ 35bp upstream of *phnG* start codon. Chromatographic analysis showed that PhnF is a dimer and has a native apparent Mr of ~27kDa which is consistent with the calculated Mr.