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Two C—P Lyase Operons in *Pseudomonas stutzeri* and Their Roles in the Oxidation of Phosphonates, Phosphite, and Hypophosphite

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DNA sequencing and analysis of two distinct C—P lyase operons in *Pseudomonas stutzeri* WM88 were completed. The *htxABCDEFGHIJKLMN* operon encodes a hypophosphite–2-oxoglutarate dioxygenase (HtxA), whereas the predicted amino acid sequences of HtxB to HtxN are each homologous to the components of the *Escherichia coli phn* operon, which encodes C—P lyase, although homologs of *E. coli phnF* and *phnO* are absent. The genes in the *htx* operon are cotranscribed based on gene organization, and the presence of the intergenic sequences is verified by reverse transcription-PCR with total RNA. Deletion of the *htx* locus does not affect the ability of *P. stutzeri* to grow on phosphonates, indicating the presence of an additional C—P lyase pathway in this organism. To identify the genes comprising this pathway, a Δ*htx* strain was mutagenized and one mutant lacking the ability to grow on methylphosphonate as the sole P source was isolated. A ca.-10.6-kbp region surrounding the transposon insertion site of this mutant was sequenced, revealing 13 open reading frames, designated *phnCDEFGHIJKLMNP,* which were homologous to the *E. coli phn* genes. Deletion of both the *htx* and *phn* operons of *P. stutzeri* abolishes all growth on methylphosphonate and aminoethylphosphonate. Both operons individually support growth on methylphosphonate; however, the *phn* operon supports growth on aminoethylphosphonate and phosphite, as well. The substrate ranges of both C—P lyases are limited, as growth on other phosphonate compounds, including glyphosate and phenylphosphonate, was not observed.

The essential nutrient phosphorus is widely held to be a redox conservative element in living systems, where it occurs only in the +5 valence state of inorganic phosphate and its organic esters, amides, and anhydrides. Nevertheless, many microorganisms are capable of metabolizing compounds containing P at lower redox states, including hypophosphite (8, 9, 22), phosphite (1, 3, 7, 8, 15, 22, 28), and phosphonic acids (for a review, see reference 30). Phosphonates, in particular, are known to be ubiquitous in nature, and in some ecosystems they comprise a major fraction of the available P (5, 10, 13). These compounds are characterized by very stable C—P bonds, in contrast to the labile C—O—P bonds found in phosphate esters. Phosphonates are produced by a variety of organisms, including both prokaryotes and eukaryotes, and can be found in the form of phosphonolipids and as side groups on polysaccharides and glycoproteins (10). In addition, a wide variety of phosphonate antibiotics are produced by microorganisms, mostly by members of the genus *Streptomyces* (29).

Given the prevalence of phosphonates in nature, it is not surprising that microorganisms have also evolved with the capacity to consume these compounds. A variety of pathways that allow specific phosphonates to be used as either the sole P or the sole carbon source have been discovered (30). The most widespread of these pathways involves the enzyme C—P lyase, which allows a variety of phosphonates to be used as sole P sources. As its name implies, C—P lyase is believed to catalyze the direct cleavage of carbon—phosphorus bonds to produce the corresponding alkane and phosphate. There is some question as to the actual products of the reaction, however, because in vitro activity has never been observed for this enzyme, despite the efforts of several research groups (4, 16, 17, 31). Thus, its products have been inferred from studies of whole or permeabilized cells.

In contrast to the dearth of biochemical data, genetic studies of *Escherichia coli* have provided considerable insight into the metabolism of P compounds by the C—P lyase pathway. A series of genetic studies revealed that a fourteen-gene operon, *phnCDEFGHIJKLMNOP,* encodes proteins required for the uptake and assimilation of phosphonates via a C—P lyase pathway (4, 19–21, 33). PhnCDE comprises a binding protein-dependent transporter with the capacity to transport not only phosphonates but also phosphate esters, phosphite, and phosphate. PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, PhnM, and PhnP are thought to comprise a multisubunit C—P lyase or, alternatively, enzymes in a multistep pathway for C—P bond cleavage. PhnF and PhnO are not required for phosphonate degradation, but are likely to be transcriptional regulators. The role of PhnN remains obscure. Although PhnN is not required for phosphonate degradation, it does appear to be involved, because *phnN* mutants grow poorly on media with phosphonates as the sole P source. PhnN was recently shown to catalyze the formation of ribose-phosphate esters (11), leading to the suggestion that the natural function of PhnN may be to produce a ribose-phosphonate ester, which may be an intermediate in phosphonate catabolism.

Surprisingly, the examination of *E. coli phn* operon mutants also revealed that these genes were required for the utilization of phosphite (P valence, +3) as the sole P source (20). Thus, in addition to its role in C—P bond cleavage, C—P lyase can also oxidize phosphite to phosphate. (The use of any P compound as the sole P source requires its conversion to phosphate,

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TABLE 1. Oligonucleotide primers used for deletion constructions

Deletion	Sequence amplified	Primer set[a]	Restriction enzyme site
Δ*htx*	Upstream *htxA*	5′-GGCGGCGGCACTAGTCTGCCCCGATATCAAGCAAC-3′	SpeI
		5′-GGCGGCGGCGCGGCCGCGTGATGCTCCAAGGTCTTCC-3′	NotI
	Downstream *htxO*	5′-GGCGGCGGCGCGGCCGCATGGCATCGAGTGCTCAAC-3′	NotI
		5′-GGCGGCGGCGAGCTCGCCGGACATTTGTATACGC-3′	SacI
Δ*ptx*	Upstream *ptxA*	5′-GGCGGCGGCACTAGTGGCTAGCATCACACAGAAACC-3′	SpeI
		5′-GGCGGCGGCGCGGCCGCCCTTGTTACCGCACTGCTTC-3′	NotI
	Downstream *ptxE*	5′-GGCGGCGCGGCCGCGGTGATGGATGGTTGCGAT-3′	NotI
		5′-GGCGGCGAGCTCCAGCGTGGCGTAGAGCTGCG-3′	SacI
Δ*phn*	Upstream *phnC*	5′-GGCGCGCCGAGCTCACCAGGCCTGGCGTGTCGGC-3′	SacI
		5′-GGCGCGCCGCGGCCGCTCTGCGCGGCCGGGGAGGT-3′	NotI
	Downstream *phnP*	5′-GGCGCGCCGCGGCCGCCGCTTAGGAGAGAAGCCGAG-3′	NotI
		5′-GGCGCGCCAACTAGTGTTCGGGCAAGCGCTCCAGC-3′	SpeI

[a] The sequences of the restriction sites incorporated into the PCR primers are underlined.

because phosphate is required for innumerable cellular processes.) Evidence of the genetic linkage between the metabolisms of different reduced P compounds was further strengthened by recent studies of the utilization of hypophosphite as the sole P source by *Pseudomonas stutzeri*.

Genetic and biochemical studies have shown that in *P. stutzeri*, hypophosphite is oxidized to phosphate via a phosphite intermediate. These reactions are catalyzed by two novel enzymes, hypophosphite–2-oxoglutarate dioxygenase and phosphite:NAD oxidoreductase (6, 22, 34), which are encoded by discrete genetic loci located ca. 15 kbp apart on the *P. stutzeri* chromosome. Phosphite oxidation in *P. stutzeri* was demonstrated to be primarily a function of the *ptxABCDE* locus, which, in addition to NAD:phosphite oxidoreductase (*ptxD*), encodes a putative binding protein-dependent phosphite transporter (*ptxABC*) and a putative transcriptional regulator (*ptxE*) (22). Partial sequencing of the *htx* locus, which encodes hypophosphite–2-oxoglutarate dioxygenase (HtxA), revealed nine open reading frames (ORFs), designated *htxABCDEF GHI′*. While *htxA* was required for hypophosphite oxidation in *P. stutzeri*, *htxBCDEFGHI′* was not. Interestingly, these genes are highly homologous to *phnCDEFGHIJ* of *E. coli*.

Given the ability of C—P lyase to oxidize phosphite to phosphate, it seemed possible that the *htx* operon could encode a complete pathway for the oxidation of hypophosphite to phosphate in a manner independent of the PtxD pathway. Thus, HtxA would oxidize hypophosphite to phosphite, and the putative *htx*-encoded C—P lyase would oxidize phosphite to phosphate. The observation that a Δ*ptx* mutant shows low levels of growth on phosphite as the sole P source after prolonged incubation is consistent with the existence of an additional phosphite oxidation pathway (A. K. White and W. W. Metcalf, unpublished results). We suspected that a complete C—P lyase operon may be present within the *htx* locus of *P. stutzeri* WM88. However, because the sequence of the *htx* locus was not complete, this hypothesis could not be verified. Moreover, strains with a deletion of the entire region encompassing both *ptx* and *htx* remained capable of growth on phosphonates, suggesting the existence of an additional, unidentified C—P lyase encoded elsewhere on the chromosome.

In this report, we show that *htx* does indeed encode a functional C—P lyase and that an additional and functional C—P lyase is present in *P. stutzeri*. Further, genetic analysis of mutants with defined deletions of two C—P lyase operons and of the *ptx* operon shows that C—P lyase can indeed play a role in phosphite and hypophosphite oxidation but not in the manner that was initially hypothesized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All *P. stutzeri* strains described are derivatives of the spontaneous streptomycin-resistant, smooth colony form of the original strain, *P. stutzeri* WM567, which in turn is a derivative of the original hypophosphite-oxidizing isolate *P. stutzeri* WM88 (22). Cloning experiments were performed with either DH5α/λpir (23) or BW20767 (18). BW20767 was also used as a donor strain in conjugation experiments. The suicide plasmid pAW19 is a derivative of pWM91 (18), which contains the BamHI kanamycin resistance cassette from pUC4K. Plasmid pWM234 (22) was used as a template for sequencing the remainder of the *htx* locus. For most experiments, Luria-Bertani broth or tryptone-yeast extract (TYE) agar plates containing the appropriate antibiotic were used (32). Kanamycin was added at 50 μg/ml, and streptomycin was added at 100 μg/ml. Selection of transformants for cloning experiments with pAW19 was done on TYE agar containing kanamycin. Selection for exconjugants harboring integrated pAW19 derivatives was done on 0.2% glucose MOPS (morpholinepropanesulfonic acid) minimal medium containing kanamycin. Sucrose-resistant recombinants resulting from segregation of pAW19 derivatives were counterselected on TYE agar with 50 g of sucrose replacing the NaCl (32).

P oxidation phenotypes were scored at 37°C on 0.2% glucose MOPS agar containing a 0.5 mM concentration of the appropriate P source. All P compounds used in this study were purchased from Sigma (St. Louis, Mo.). The purity of some P compounds with respect to contaminating P-containing compounds was assessed by ^{31}P nuclear magnetic resonance as described in reference 34. Compounds that failed to support growth were assumed to be free of contaminating levels of phosphate. All compounds were greater than 99% pure with respect to their P content. When glyphosate (phosphonomethylglycine) was used as the P source, the media were supplemented with 0.05 mg of an aromatic-amino-acid

TABLE 2. Oligonucleotide primers used for the amplification of *htx* junction sequences

Junction amplified	Primer set	Predicted product size (bp)
htxAB	5′-AAGTGTACGACCACCGGAAC-3′	586
	5′-GTCTGGCTAGGCGTTGGAC-3′	
htxBC	5′-TCCTTCGAAGTCCAAGATCG-3′	587
	5′-CAGCCCCTTTTAGAATGACG-3′	
htxCD	5′-CAGCAGGGCAAAGATTATCC-3′	560
	5′-CCGGAGACAGTTCATGTATGG-3′	
htxDE	5′-GTGCATGCGCTTTACCAG-3′	583
	5′-ACAGCGCAATCTCCAGGTCT-3′	
htxEF	5′-AGAACGCCGACAAGAAGC-3′	589
	5′-TTCGATCGACTGCCCTTG-3′	
htxFG	5′-GCATGCCAGGTTGTGAAG-3′	573
	5′-GCACCTGCTCGTGTTGAATA-3′	
htxGH	5′-GGCAACCGTTTGCTGGAG-3′	581
	5′-CACCGGCACATCCATCAG-3′	
htxHI	5′-CCGGTGAGGTACTGATGACC-3′	553
	5′-AATCAGCGACAGCGTAACCT-3′	
htxIJ	5′-CATCAACGGCCACTATGTGA-3′	700
	5′-CTGGTACACCATGCCAAAGC-3′	
htxJK	5′-CCGCTGCTCTACCTCGAC-3′	591
	5′-ACAGTGCAGAAATTGGGTGA-3′	
htxKL	5′-GAACTGCTGGCCCTGCTC-3′	556
	5′-AACTCCAGCGGGAAGTGC-3′	
htxLM	5′-CTGCCTGTGCGGTGACTACT-3′	600
	5′-GGGCCTCGTCCAGGTATT-3′	
htxMN	5′-GAATCGGCATCGAGATCAAC-3′	584
	5′-CAGCGCAGATGAAAGAGTCC-3′	
htxN-orf282	5′-GCTTCGCCTACCTCACAGAC-3′	587
	5′-AACTGCGCACCAGAGGATAG-3′	
orf282-orf344	5′-CTCTTGGCGGAACCTGTATC-3′	569
	5′-GAGCGAATGCCTTTGAGAAC-3′	

mixture/ml. To remove the contaminating phosphate, the agar and all glassware were rinsed with multiple changes of ultrapure deionized water prior to use. The solutions of all P compounds were made immediately prior to use and were filter sterilized.

DNA methods. Standard methods were used for the isolation and manipulation of plasmid DNA. Chromosomal DNA was isolated from *P. stutzeri* strains as described previously (2). DNA hybridizations were carried out by using a digoxigenin system as recommended by the manufacturer (Roche, Mannheim, Germany). Sequencing reactions were performed with the BigDye sequencing reagent (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer and were analyzed at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana.

Plasmid construction. Plasmid pJB1 carries a deletion of *htxA-htxO* in a pAW19 vector backbone. To create the deletion, ca. 1.0 kbp of sequence upstream of the *htxA* translational start site and ca. 1.0 kbp of sequence downstream of *htxO* were amplified by PCR using *Taq* DNA polymerase and the primers listed in Table 1. The PCR products were digested with the appropriate restriction enzymes and ligated to the SpeI and SacI sites of pAW19.

Plasmid pJB2 carries a complete deletion of both the *ptx* and *htx* operons (*ptxA-orf344*) in a pAW19 vector backbone. To create this deletion, ca. 1.0 kbp of sequence directly upstream of the *ptxA* translational start site was amplified by PCR with *Taq* DNA polymerase and the primers listed in Table 1. The resulting PCR fragment was digested with SpeI and NotI and was inserted into the same sites of pJB1, resulting in the replacement of the *htxA* upstream fragment with the *ptxA* upstream fragment.

Plasmid pAW52 carries a *phnC-phnP* deletion in a pAW19 vector backbone. To construct the deletion, ca. 1.0 kbp of sequence upstream of the *phnC* translational start site and 1.0 kbp of sequence directly downstream of the *phnP* stop codon were amplified by PCR with *Pfu* Turbo DNA polymerase and the primers listed in Table 1. The resulting PCR products were digested with the appropriate restriction enzymes and were inserted between the SpeI and SacI sites of pAW19.

Plasmid pAW79 carries a *ptxA-ptxE* deletion. To construct this deletion, the 1.0 kbp of sequence upstream of the *ptxA* translational start site and the 1.0 kbp of sequence downstream of the *ptxE* stop codon were amplified by PCR with *Pfu* Turbo DNA polymerase and the primers listed in Table 1. The resulting PCR products were digested with the appropriate restriction enzymes and were inserted between the SpeI and SacI sites of pAW19.

Genetic techniques. The in vitro-constructed deletions were recombined onto the chromosome of *P. stutzeri* WM567 by *sacB* counterselection as previously described (18). The conjugative transfer of plasmids from *E. coli* BW20767 to *P. stutzeri* WM567 was done by using the filter mating technique as previously described (14), with selection of exconjugants on 0.2% glucose MOPS medium containing 0.5 mM phosphate and kanamycin. Sucrose-resistant segregants were screened for loss of the integrated plasmid by scoring their kanamycin sensitivity. The correct mutant constructs were differentiated from wild-type segregants by DNA hybridization analysis using as a probe the same plasmid used to create the deletion.

Isolation and cloning of the *phn* genes of *P. stutzeri* was done in a strain of *P. stutzeri* WM1926 (*rpsL* Δ*ptxA-orf344*) by in vivo Tn*5* mutagenesis with pRL27 as previously described (14). Transposon mutants were screened for the inability to grow on glucose-MOPS medium with methylphosphonate as the sole source of P. The BamHI chromosomal fragment containing the Tn*5* insertion site and flanking region was cloned to create plasmid pMW5. This plasmid carries the *phnI*::Tn*5*-RL27 insertion along with a substantial region of flanking DNA and was used as a sequencing template for the *phn* locus of *P. stutzeri*.

RT-PCR. Total RNA was isolated from cultures of *P. stutzeri* grown to mid-logarithmic stage (optical density at 600 nm of ca. 0.6) in 0.2% glucose MOPS minimal medium with 0.5 mM hypophosphite as the sole source of P. RNA isolation was carried out with an RNeasy mini kit with RNAprotect bacterial reagent (QIAGEN, Inc., Valencia, Calif.) per the manufacturer's instructions. To remove contaminating chromosomal DNA, the RNA preparation was digested with amplification grade DNase I (Invitrogen, Carlsbad, Calif.). The DNase I-treated RNA was then used as a template in a reverse transcription (RT) assay using SuperScript II RNase H^- reverse transcriptase (Invitrogen), according to the manufacturer's protocol. PCR amplification of the cDNA from the RT reaction was performed with Platinum *Pfx* DNA polymerase (Invitrogen) as recommended. A positive control, in which only chromosomal DNA was added to the PCR, and a negative control, in which only RNA without reverse transcriptase was used for the PCR, were run under identical PCR amplification conditions. The primers used are listed in Table 2.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *P. stutzeri* WM88 DNA sequences determined in this study are AF061267 for the completed *htxIJKLMN* and *orf282-orf344* sequences and AY505177 for the complete *phnCDEFGHIJKLMNP* sequence.

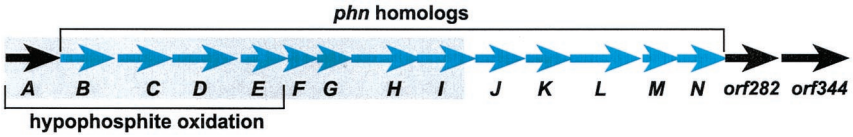

FIG. 1. Structure of the *htx* operon of *P. stutzeri* WM88. Black arrows indicate genes with no homology to *phn* genes. Blue arrows indicate *htx* genes that are homologous to *phn* genes, which comprise all of the components of a complete C—P lyase. The sequences of the genes lying within the shaded gray box had previously been determined (22).

RESULTS

Complete sequence analysis of the *htx locus*. A previously isolated cosmid clone (22) harboring a fragment of *P. stutzeri* chromosomal DNA containing *htxA* to *htxI* and downstream sequence was used as a template to obtain the complete sequence of the *htx* locus beyond *htxI′*. Immediately downstream of the nine previously identified ORFs (*htxA-htxI*) (22), seven additional ORFs were observed. These were designated *htxJKLMN*, *orf282*, and *orf344* (Fig. 1).

FASTA searches with the predicted amino acid sequences of HtxB to ORF344 (27) were performed against sequences in the nonredundant Swiss Protein Database. HtxB to HtxN are each homologous to the corresponding PhnC to PhnP components of the *E. coli* C—P lyase, except that homologs to PhnO and PhnF, which are not required for phosphonate utilization in *E. coli* (21), are absent (Table 3). Thus, all of the genes required to encode a functional C—P lyase are present in a 10.9-kbp region immediately downstream of HtxA. The *htx* operon not only is homologous to the *E. coli phn* operon at the level of the predicted amino acid sequences but also conserves the order of the *E. coli* C—P lyase genes (minus those that are absent). The downstream *orf282* and *orf344* genes do not encode Phn-like proteins but instead are homologous to a hypothetical transmembrane protein and a putative cointegrate resolution protein, respectively (Table 3).

The *htxA* gene and the putative C—P lyase-encoding genes comprise a single transcriptional unit. The close proximity and/or overlap of the ORFs within the *htx* locus suggest that they form a single transcriptional unit. Of the 14 ORFs identified, 5 have coding regions that overlap, whereas the remaining ORFs are separated by, at most, 44 nucleotides, with the exception of *htxBC*, which is separated by 125 nucleotides.

To test whether these genes are cotranscribed, RT-PCR was used to determine the presence of the junction sequences between each gene in total RNA isolated from hypophosphite-grown *P. stutzeri* (Fig. 2). Amplification products were seen for the intergenic regions between all adjacent *htx* genes, with the exception of *htxHI*. Nevertheless, because *htxH* and *htxI* overlap by 5 bp, it is likely that they are cotranscribed. Therefore, the *htxABCDEFGHIJKLMN* genes almost certainly comprise an operon. Amplification products were not observed for the regions between the *htxN*, *orf282*, and *orf344* genes, which, in conjunction with their lack of homology to the known P assimilation genes, suggests that they are not within the same transcriptional unit as the *htx* genes.

Identification of a second C—P lyase-encoding locus in *P. stutzeri*. To investigate whether *htxB* to *htxN* encode a functional C—P lyase, and to determine the role of this putative C—P lyase with respect to phosphite and phosphonate metabolism, a strain with a deletion of the entire region encompassing the *htx* and *ptx* operons (Δ*ptxA-orf344* mutant) was constructed. Consistent with previous results (22), the deletion mutant was no longer able to grow on hypophosphite; however, growth on methylphosphonate and aminoethylphosphonate was still observed. Furthermore, after prolonged incubation, a very low level of growth was observed on phosphite as the sole P source. These data suggest the presence of an additional pathway(s) for the utilization of phosphonates and phosphite.

The genes permitting growth on phosphonates and phosphite in the Δ*ptxA-orf344* strain were identified by in vivo Tn*5*-RL27 transposon mutagenesis as described previously (14). A Tn*5* insertion mutant unable to grow on methylphosphonate or phosphite as the sole P source was isolated, and the sequence with mutation was cloned. The sequence adjacent to the Tn*5*-RL27 insertion showed that the transposon was inserted into an *E. coli phnI* homolog. Additional sequencing of the flanking DNA revealed the presence of 13 ORFs that also share significant predicted amino acid sequence identity with putative Phn proteins of *Pseudomonas aeruginosa* and *Pseudomonas syringae* (Table 3). Both the sequence and the organization of the 13 ORFs are homologous to the genetically characterized *phn* operon of *E. coli*, except that *phnO* is absent from the *P. stutzeri phn* sequence. The inability of the Δ*ptxA-orf344 phnI*::Tn*5*-RL27 mutant to utilize methylphosphonate as a sole P source, in contrast to its parent, indicates that the *P. stutzeri phnCDEFGHIJKLMNP* operon encodes a functional C—P lyase.

Interestingly, the genes constituting this operon are distinctly different from the *phn*-like genes *htxB* to *htxN*. Pairs of predicted amino acid sequences were aligned to compare the *P. stutzeri htx*-encoded C—P lyase components with the corresponding *P. stutzeri phn*-encoded C—P lyase subunits (Table 3). The *P. stutzeri* Phn proteins share significant identity with the Phn proteins of other pseudomonads (61 to 90% identity) and with *E. coli* (39 to 75%) but share much less identity with the *htx*-encoded Phn-like proteins (20 to 53%), which are generally more similar to the Phn components of bacteria from the family *Rhizobiaceae*. This trend is also true with respect to the organization of the genes in the *htx* and *phn* operons of *P. stutzeri* in comparison to that of *E. coli* (Fig. 3). The *P. stutzeri phn* operon is quite similar to that of *E. coli*, missing only a homolog to *E. coli phnO*. In contrast, the *htx* operon not only contains an additional *phnE* homolog, but also is missing homologs to the putative regulators encoded by both *phnF* and *phnO*.

The roles of the *htx* and *phn* operons in the oxidation of reduced P compounds. The roles of the *phnC* to *-P* and *htxA* to *-N* operons in the metabolism of reduced P compounds were

TABLE 3. Comparison of the Htx and Phn proteins in *P. stutzeri* with each other and with the Phn proteins of *E. coli*

Phn protein	Closest homolog of predicted product (% identity)[a]	% Identity to *E. coli* Phn homolog[b]	Htx protein	Closest homolog of predicted product(s) (% identity)	% Identity to *E. coli* Phn homolog[b]	Phn and Htx homologs of *P. stutzeri* WM88[c]	% Identity between Phn and Htx proteins	Proposed function of Phn and Htx proteins
PhnD	*P. aeruginosa* (PAO1) PhnD (73.7)	54.0	HtxB	*Nostoc* sp. PhnD, phosphonate binding protein (25.7)	18.1	PhnD/HtxB	20.3	Phosphonate binding protein of ABC transporter
PhnE	*P. aeruginosa* (PAO1) PhnE (85.9)	69.2	HtxC	*Bradyrhizobium japonicum* PhnE (40.1)	32.6	PhnE/HtxC	33.3	Phosphonate transport permease
			HtxE	*Bacillus anthracis* PhnE (41.3)	26.8	PhnE/HtxE	29.8	
PhnC	*P. syringae* (pv. tomato) PhnC (80.8)	58.3	HtxD	*E. coli* PhnC (44.7)	44.7	PhnC/HtxD	20.3	Phosphonate binding protein of ABC transporter
PhnF	*P. aeruginosa* (PAO1) probable transcriptional regulator (67.9)	41.1	—[d]					Transcriptional regulator (GntR family)
PhnG	*P. syringae* (pv. tomato) PhnG (73.2)	43.9	HtxF	*Sinorhizobium* sp. PhnG (33.3)	21.4	PhnG/HtxF	22.6	Phosphonate metabolism protein
PhnH	*P. syringae* (pv. tomato) PhnH (61.0)	33.2	HtxG	*E. coli* PhnH (32.6)	32.6	PhnH/HtxG	25.4	Phosphonate metabolism protein
PhnI	*P. syringae* (pv. tomato) PhnI (80.8)	62.9	HtxH	*Mesorhizobium loti* PhnI (45.2)	43.6	PhnI/HtxH	42.6	Phosphonate metabolism protein
PhnJ	*P. syringae* (pv. tomato) PhnJ (89.5)	75.4	HtxI	*E. coli* PhnJ (53.7)	53.7	PhnJ/HtxI	53.0	Phosphonate metabolism protein
PhnK	*P. aeruginosa* (PAO1) putative ATP binding component (86.3)	65.2	HtxJ	*Agrobacterium tumefaciens* PhnK (40.2)	32.3	PhnK/HtxJ	30.5	Phosphonate ATP binding protein
PhnL	*P. aeruginosa* (PAO1) putative ATP binding component (86.0)	58.5	HtxK	*M. loti* PhnL (52.0)	42.6	PhnL/HtxK	42.6	Phosphonate ATP binding protein
PhnM	*P. syringae* (pv. tomato) PhnM (71.6)	56.5	HtxL	*Rhizobium luguminosarum* PhnM (36.6)	34.7	PhnM/HtxL	35.0	Phosphonate metabolism protein
PhnN	*P. syringae* (pv. tomato) PhnN (62.0)	39.4	HtxM	*S. meliloti* PhnN (47.3)	41.6	PhnN/HtxM	37.4	Phosphonate metabolism accessory protein
PhnP	*P. syringae* (pv. tomato) PhnP (71.5)	46.0	HtxN	*P. aeruginosa* conserved hypothetical protein (53.0); *P. syringae* PhnP (51.0)	45.9	PhnP/HtxN	49.0	Phosphonate metabolism accessory protein

[a] Closest homologs were determined with FASTA searches of the predicted amino acid sequences against sequences in the nonredundant Swiss Protein Database on 15 May 2003.
[b] Pairwise sequence alignments were performed with the predicted amino acid sequences of the *P. stutzeri* Phn or Htx protein with the *E. coli* Phn homologs.
[c] Pairwise sequence alignments were performed with the predicted amino acid sequences of the *P. stutzeri* Phn or Htx proteins.
[d] The Phn homolog was absent from the *htx* sequence.

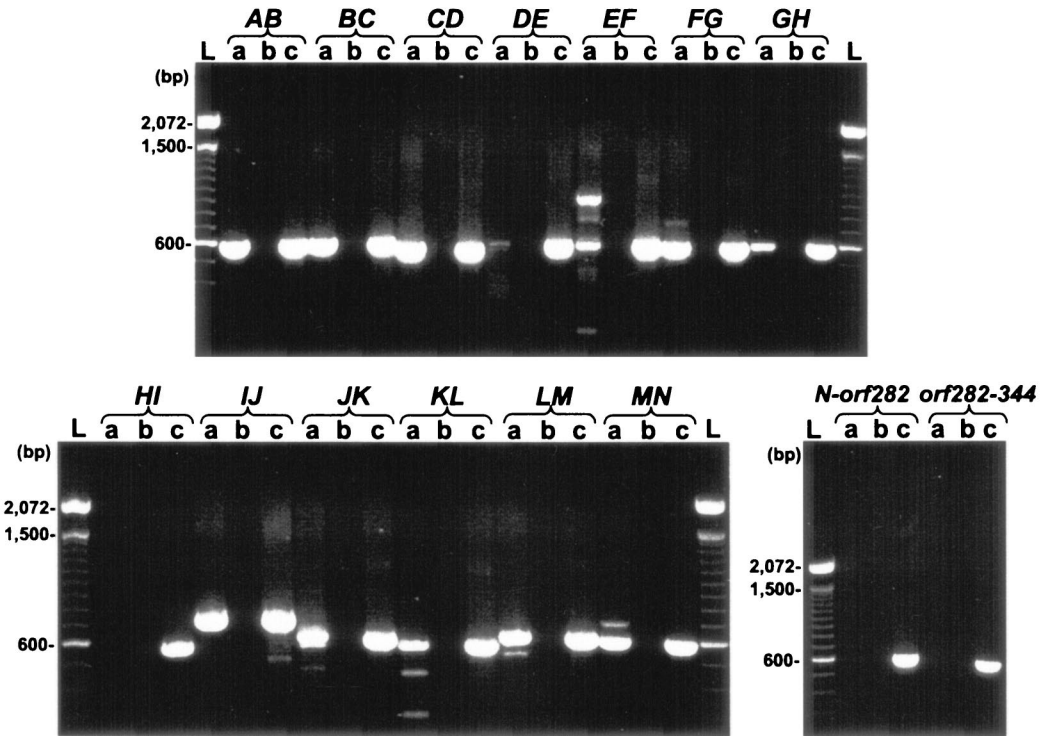

FIG. 2. RT-PCR of total RNA prepared from *P. stutzeri* WM567 grown on hypophosphite as the sole P source to determine the operon structure of *htx*. Lanes a show complete RT reactions; lanes b contain a negative control, with which no reverse transcriptase was added to the reaction mixture; and lanes c contain a PCR-positive control, for which chromosomal DNA was used as the template. Lanes L, 100-bp ladder. The junction sequences amplified are indicated above each set of reactions. For a list of the primers used and the predicted PCR product sizes, refer to Table 2.

further examined using a series of mutants harboring complete deletions of *phnC* to *phnP* (Δ*phn*), *ptxA* to *ptxE* (Δ*ptx*), and/or *htxA* to *orf344* (Δ*htx*) as single, double, or triple mutants. Each mutant was tested for its ability to utilize a variety of different reduced P compounds as sole P sources (Table 4).

Growth of the Δ*phn* Δ*ptx* strain on methylphosphonate as the sole source of P indicates that the putative C—P lyase system encoded by the *htx* operon is functional. However, the growth conferred by the *htx*-encoded C—P lyase is poor compared to that of the Δ*htx* Δ*ptx* strain, which relies on the *phn*-encoded C—P lyase system for methylphosphonate utilization (Fig. 4, compare streaks 5 and 6). Also, the *htx*-encoded system confers growth on methylphosphonate but does not allow growth on aminoethylphosphonate, whereas the *phn*-encoded C—P lyase confers growth on both substrates. The substrate specificities of both *P. stutzeri* C—P lyase systems are

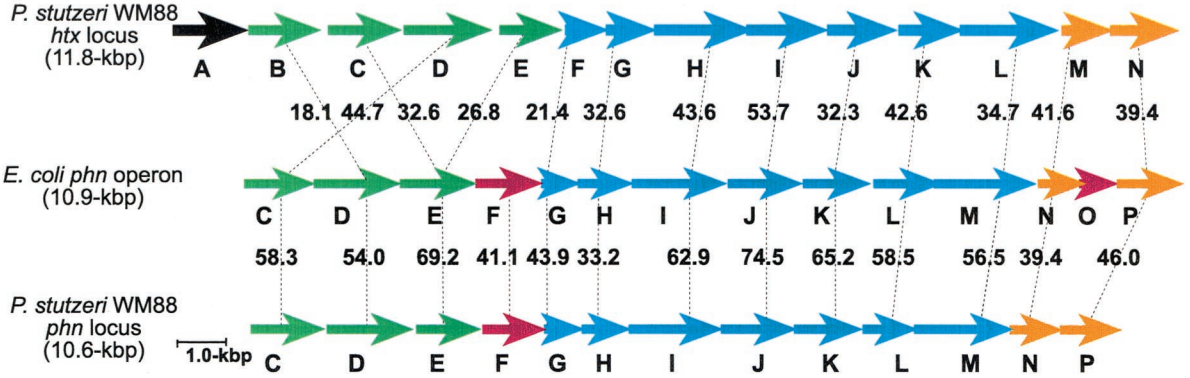

FIG. 3. Organization of the genes of the *htx* and *phn* operons involved in the metabolism of phosphonates in *P. stutzeri* compared to that of the *phn* operon of *E. coli*. The black arrow represents genes within the *htx* operon that are not homologous to any *phn* gene, green arrows represent genes likely involved in phosphonate transport, red arrows represent genes with putative regulatory function, blue arrows represent genes thought to encode the catalytic components of the C—P lyase, and gold arrows represent genes believed to encode accessory proteins to C—P lyase. The percentages of predicted amino acid sequence identity between each of the homologous proteins are indicated.

TABLE 4. Growth of *P. stutzeri* Δ*htx*, Δ*ptx*, and Δ*phn* mutants on various P sources

Phosphorus source	Growth[a]							
	WM567 (WT)	WM1926 (Δ*htx*)	WM3746 (Δ*ptx*)	WM3614 (Δ*phn*)	WM3747 (Δ*htx* Δ*ptx*)	WM3748 (Δ*phn* Δ*ptx*)	WM3616 (Δ*htx* Δ*phn*)	WM3617 (δ*ptx-htx* Δ*phn*)
Phosphate	+++	+++	+++	+++	+++	+++	+++	+++
Phosphite	+++	+++	+[b]	++	+/−[b]	−	+++	−
Hypophosphite	+++	+/−	+[b]	+++	+/−[b]	−	+/−	−
Methylphosphonate	++	+++	++	+	+++	+	−	−
Aminoethylphosphonate	++	+++	++	−	+++	−	−	−
Phenylphosphonate	−	−	−	−	−	−	−	−
Phenylphosphinate	−	−	−	−	−	−	−	−
Dimethylphosphinate	−	−	−	−	−	−	−	−
Glyphosate	−	−	−	−	−	−	−	−
Phosphomycin	−	−	−	−	−	−	−	−

[a] +++, growth equal to that of the positive-control strain WM567 on 0.5 mM K_2HPO_4 after 2 days of incubation at 37°C; ++, growth equal to that of the positive control after 4 days of incubation at 37°C; +, growth equal to that of the positive control after 7 days of incubation at 37°C; +/−, growth exceeding that of the negative control but not equal to that of the positive control after 7 days of incubation at 37°C; −, growth equal to that of the negative-control strain WM567 on phosphate-free agar without an added P source after 7 days of incubation at 37°C. WT, wild type.

[b] Mutant colonies grew above the background growth for this sample.

rather narrow relative to those of other known C—P lyase systems (26, 31); no growth was observed by any of the strains on phenylphosphonate, phenylphosphinate, dimethylphosphinate, glyphosate, or phosphonomycin, even after an incubation time of greater than 10 days. These differences in substrate utilization may reflect the specificity of the C—P lyase enzyme and/or the associated phosphonate transport systems or, alternatively, may reflect differences in expression of the two operons in response to different substrates. Deletion of both *htx* and *phn* abolishes growth on all phosphonates, indicating that these are the only two pathways present in *P. stutzeri* for the utilization of phosphonates.

As previously observed, strains with the *ptx* genes deleted retain a small amount of growth on phosphite after 7 days of incubation. This growth is absent in the Δ*ptx* Δ*phn* mutant, consistent with a role for *phn* in phosphite oxidation, as has been previously described for *E. coli* (20). Interestingly, the lack of growth on phosphite for the Δ*ptx* Δ*phn* mutant also demonstrates that the *htx*-encoded C—P lyase is incapable of phosphite oxidation.

Strains that rely on *phn* for phosphite oxidation give rise to better-growing mutants during growth with phosphite or hypophosphite as the sole P source (Fig. 4B and C). The responsible mutations are most likely within the *phn* operon, because such mutations were not observed in Δ*phn* strains. Interestingly, the absence of similar mutations in the Δ*phn* Δ*ptx* mutant suggests that the *htx* operon cannot be mutated to allow phosphite oxidation. Strains in which *ptx* remains intact also do not give rise to better-growing mutants; however, *ptx*-dependent growth on phosphite is much more robust than *phn*-dependent growth.

Surprisingly, it appears that the *htx*-encoded C—P lyase interferes with *phn*-encoded phosphonate utilization. Thus, strains from which the *htx* operon is deleted grow faster on phosphonates than does the wild type (Fig. 4, streak 3 or 6 compared to streak 4). A similar effect is not observed in Δ*phn* strains, indicating that the *phn*-encoded C—P lyase does not negatively impact the *htx*-encoded C—P lyase. An even more pronounced effect is observed when plasmids with the *P. stutzeri htx* operon are carried in *E. coli*. In this case, *phn*$^+$ *E. coli* strains become completely incapable of phosphonate utilization (data not shown). This negative interaction is specific for growth on phosphonates and is not observed during growth on phosphite. Instead, the opposite was observed; i.e., growth on phosphite was better in the presence of the *htx* operon than in its absence (compare streak 7 to streak 6), despite the observation that the presence of *htx* alone did not allow the strain to grow on phosphite. This result suggests that some subset of genes with the *htx* operon can function in concert with *phn* operon products to promote phosphite oxidation.

DISCUSSION

The data presented here show that *P. stutzeri* encodes two discrete C—P lyases that allow the use of phosphonates as sole P sources. Although the presence of multiple C—P lyases within the same host has previously been suggested based on the assimilation kinetics of different phosphonic acid substrates (12), this is the first case in which multiple operons have been definitively identified and phenotypically characterized. Interestingly, there are significant differences between the *htx*-encoded and *phn*-encoded C—P lyases. The proteins encoded by the *phn* and *htx* operons are more similar to homologs from other bacteria than they are to each other, with the *phn* gene products being most similar to homologs from other pseudomonads (62 to 90%) and the *htx* operon being more similar to homologs found in members of the *Rhizobiaceae* (33 to 52%). These data strongly suggest that the two operons evolved in different hosts, rather than by duplication of the same operon within the chromosome. Further, given the homology of the *phn* operon to those of other pseudomonads, it seems likely that the *htx* operon was the more recent addition to the *P. stutzeri* genome.

Additional differences were observed in the substrate specificities of the two C—P lyase systems. The *phn*-encoded C—P lyase system allows growth on methylphosphonate, aminoethylphosphonate, and phosphite, while the *htx*-encoded C—P lyase system allows growth only on methylphosphonate. Both systems have relatively narrow substrate profiles relative to those of other known C—P lyase systems: neither operon al-

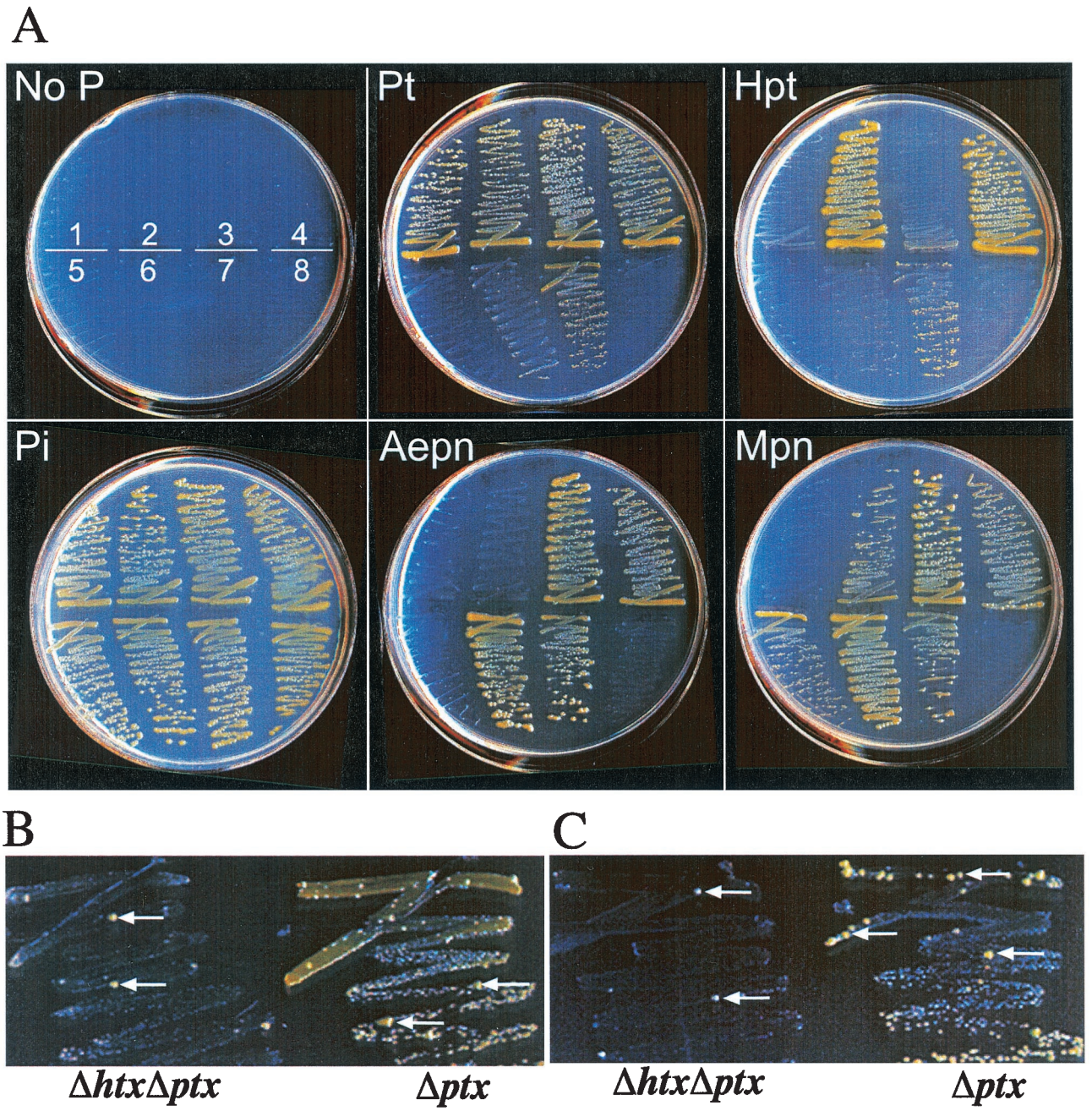

FIG. 4. Growth of *P. stutzeri htx*, *ptx*, and *phn* deletion mutants on minimal media with various P sources after 4 days of growth. (A) Fresh cultures were streaked from MOPS minimal solid agar containing 0.1 mM P_i onto MOPS minimal agar containing the indicated P sources (phosphite [Pt], hypophosphite [Hpt], inorganic phosphate [Pi], aminoethylphosphonate [Aepn], and methylphosphonate [Mpn]) at 0.5 mM. The schematic on the plate lacking phosphorus (No P) represents the order in which the deletion mutants were streaked: 1, Δ*htx* Δ*phn* mutant (WM3616); 2, Δ*phn* mutant (WM3614); 3, Δ*htx* mutant (WM1926); 4, wild type (WM567); 5, Δ*phn* Δ*ptx* mutant (WM3748); 6, Δ*htx* Δ*ptx* mutant (WM3747); 7, Δ*ptx* mutant (WM3746); and 8, Δ*ptx-htx* Δ*phn* mutant (WM3617) (B and C). Representative Δ*htx* Δ*ptx* and Δ*ptx* mutant colonies showing enhanced growth that were grown on phosphite (B) and hypophosphite (C) are indicated by white arrows.

lowed growth on phenylphosphonate, glyphosate, or various phosphinates, although these substrates are widely used by other bacteria (26, 31). It is important to note that this substrate specificity is not necessarily a property of the C—P lyase enzyme. Differences in substrate specificity of the associated phosphonate transporters could also account for these data, as could differences in the expression of the two operons in response to different substrates.

Further evidence that *phn* and *htx* evolved in different organisms stems from the observation that the *htx* operon has a negative effect on the function of the *phn*-encoded C—P lyase. This negative interaction appears to be one-sided, in that de-

letion of *htx* improves growth on phosphonates conferred by *phn* but deletion of *phn* does not improve growth on methylphosphonate conferred by *htx*. Further, the negative effect of *htx* is not specific for the *P. stutzeri phn* operon and is even more pronounced in *E. coli*. Although the nature of this negative interaction is unknown, there are several possible explanations for this phenotype. It has been postulated that C—P lyase is a membrane-associated enzyme complex. This supposition is supported by the observation that C—P lyase activity in whole-cell suspensions does not require *phnCDE* for phosphonate transport (35). Given the likelihood that the components of C—P lyase interact in some way to form an active complex, it seems possible that the respective Htx and Phn proteins are similar enough to each other to form hybrid, inactive complexes. Such negative interactions may also exist in the assembly of the binding protein-dependent transporters encoded by *htxBCDE* and *phnCDE*. The existence of dominant negative mutants incapable of assembling the homologous *E. coli* maltose transport system suggests that such a scenario is possible (24). Similar interactions between *htxBCDE* and *phnCDE* might result in the formation of a hybrid, inactive transport system, resulting in deficient phosphonate uptake. Careful analysis of the nature of this dominant negative effect may significantly add to our understanding of the C—P lyase reaction, revealing how the many proteins required for catalysis and/or transport interact with one another.

We had anticipated that both the *htx*- and *phn*-encoded C—P lyases would allow phosphite oxidation, as does the C—P lyase of *E. coli*; however, our data indicate that only the *phn*-encoded C—P lyase has this property. Despite this ability, we conclude that phosphite oxidation is predominantly a function of the *ptx* operon, in agreement with the results of a previous study (22). Thus, *phn*-dependent growth on phosphite is much poorer than *ptx*-dependent growth. Further, *ptx* deletion strains, which depend on the *phn* operon for phosphite oxidation, give rise to mutants with better growth on hypophosphite or phosphite (Fig. 4B and C). This finding suggests that the *phn* operon has not been subject to selective pressure for better phosphite utilization since the acquisition of the *ptx* operon. In this context, it should be noted that, while most pseudomonads possess a *phn* operon (based on available genomic sequences), the *htx* and *ptx* loci appear to be uncommon and are more likely to be recent acquisitions. Thus, although pseudomonads with these genes are easily isolated by selective enrichment, none of the published genomes contain these genes, nor do six authentic *P. stutzeri* strains from the American Type Culture Collection (22).

The observation that the *htx* operon does not allow growth on phosphite contradicts our hypothesis that this operon encodes a complete pathway for hypophosphite oxidation. Nevertheless, our data do suggest a potential linkage between the use of hypophosphite and phosphonates, because the *htx*-encoded C—P lyase is cotranscribed with the hypophosphite-oxidizing enzyme encoded by *htxA*. Three explanations for this linkage seem plausible. First, our original hypothesis may have been correct at an earlier stage in evolution. Accordingly, it is possible that the *htx* operon once provided phosphite oxidation activity and that over time, more-efficient pathways for the oxidation of phosphite, such as *ptx* and *phn*, were introduced in *P. stutzeri*. Alternatively, a phosphite-oxidizing *htx* operon may have been introduced into a strain that already possessed *phn* and/or *ptx*. In either case, this introduction may have resulted in the loss of the phosphite oxidation phenotype conferred by *htx* due to the lack of selective pressure to maintain it. Moreover, the negative interaction between the two C—P lyases may have provided additional pressure for loss-of-function mutations in the *htx* operon. A second explanation suggests that the *htx* operon encodes a two-step pathway for the oxidation-reduced P compounds having both carbon—phosphorus and hydrogen—phosphorus bonds, such as (C—P—H) phosphinates (P valence, +1). One such compound, demethylphosphinothricin, is known to be produced by several *Streptomyces* species during the synthesis of the antibiotic bialaphos and is likely present in the soil (25). Such a pathway would involve the oxidation of the C—P—H bonds by HtxA to produce a phosphonate (C—P—OH), which would then be oxidized to phosphate by the *htx*-encoded C—P lyase. Unfortunately, our attempts to test this idea by examining growth on two commercially available phosphinates, dimethylphosphinate and phenylphosphinate, failed due to the lack of growth on either substrate. Nevertheless, as-yet-unidentified phosphinate substrates for this putative HtxA/C—P lyase pathway may exist. Finally, it may be that HtxA and C—P lyase are cotranscribed only because both pathways are used to acquire P from less favorable sources and, thus, are regulated by a common phosphate starvation-inducible promoter. Their close proximity may simply be due to a grouping of related genes whose expression is under the control of the same regulators.

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