JOURNAL OF BACTERIOLOGY, Sept. 2004, p. 5876–5882 Vol. 186, No. 17
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.17.5876–5882.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

The *htx* and *ptx* Operons of *Pseudomonas stutzeri* WM88 Are New Members of the Pho Regulon

Andrea K. White and William W. Metcalf*

Department of Microbiology, University of Illinois, Urbana, Illinois

Received 12 April 2004/Accepted 7 June 2004

The *htx* and *ptx* operons of *Pseudomonas stutzeri* WM88 allow for the use of the inorganic reduced phosphorus (P) compounds hypophosphite (P valence, +1) and phosphite (P valence, +3) as sole P sources. To support the proposed in vivo role for the *htx* and *ptx* operons, namely the use of phosphite and hypophosphite as alternative P sources, we used reporter gene fusions to examine their expression levels with respect to various P conditions. Expression of the *htx* and *ptx* operons was induced up to 17- and 22-fold, respectively, in cultures grown under phosphate starvation conditions relative to expression in medium with excess phosphate (P_i). However, the presence of the reduced P substrate hypophosphite, phosphite, or methylphosphonate, in addition to excess P_i, did not result in an increase in the expression of either operon. To provide further support for a role of the *htx* and *ptx* operons in P_i acquisition, we identified *P. stutzeri phoBR* homologs and constructed deletion mutants. Induction of the *htx* and *ptx* reporter gene fusions in response to growth on limiting P_i was abolished in Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutants, demonstrating that *htx* and *ptx* expression is *phoBR* dependent. The putative LysR-type regulator encoded by *ptxE* has no apparent role in the expression of the *htx* and *ptx* operons, as no effect was observed on the level of induction of either operon in a Δ*ptxE* mutant.

Despite the fact that phosphorus has long been considered the only essential element that does not partake in biologically catalyzed oxidation-reduction reactions, it has become increasingly evident that utilization of the inorganic reduced phosphorus compounds hypophosphite (P valence, +1) and phosphite (P valence, +3) as alternative phosphorus sources is common among microorganisms. Microbial oxidation of hypophosphite and phosphite has been documented in the literature for several decades (1, 6, 10, 12, 17, 19). Although these studies clearly established the microbial oxidation of these compounds, the processes by which this occurs remained largely unexplored in any detail on the genetic or biochemical level, until recently.

A genetic analysis of hypophosphite oxidation in *Pseudomonas stutzeri* WM88 led to the identification of two distinct regions of the chromosome, *htxABCDEFGHIJKLM* and *ptxABCDE*, that are required for the oxidation of hypophosphite and phosphite, respectively (20). Subsequent purification and biochemical characterization of the putative P-oxidizing enzymes HtxA and PtxD demonstrated that the two enzymes form a biochemical pathway for the oxidation of hypophosphite to P_i (5, 34). Genetic and biochemical data support the hypothesis that the *htx* and *ptx* genes serve the purpose of providing the organism with alternative sources of phosphorus.

In many bacteria, genes involved in the assimilation of P_i from various phosphorus compounds in the environment are phosphate starvation inducible (Psi). Collectively, such genes comprise a phosphate (Pho) regulon that is controlled by the two-component signal transduction system PhoBR (13–16, 30). The Pho regulon of *Escherichia coli*, for example, includes genes that encode transport systems for the uptake of P_i and a variety of alternate phosphorus sources, such as organophosphates and phosphonates, as well as genes that encode enzymes required for the utilization of alternative phosphorus sources (*pstSCAB*, *ugpBAEC*, *phoA*, and *phnC-phnP*) (2, 27, 33, 36). Under conditions of P_i starvation, phosphorylated PhoB binds to a highly conserved sequence called a Pho box located within the promoters of the genes that it activates (30). Although considerable data exist regarding the regulation of the *E. coli phn* genes required for the use of phosphonates (P valence, +3), no information is yet available on the regulation of genes required for the utilization of other reduced phosphorus compounds such as hypophosphite and phosphite.

Although the genes within the *htx* and *ptx* operons are clearly responsible for hypophosphite and phosphite oxidation in *P. stutzeri*, the physiological relevance of such a process with respect to phosphorus acquisition is less clear. To our knowledge, neither hypophosphite nor phosphite has ever been measured in the natural environment. However, note that a recent study demonstrated that previously used methods were inadequate for this task (22). To further clarify the in vivo role of the *htx* and *ptx* operons in *P. stutzeri* with respect to the oxidation of these compounds, we examined the regulation of expression of both operons. Here we report an expression analysis of *htx* and *ptx* in response to P_i starvation in *P. stutzeri* and demonstrate the dependence of this expression on *phoBR*, supporting a role for these genes in phosphorus acquisition through the oxidation of hypophosphite and phosphite.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used for this study are shown in Table 1. *E. coli* DH5αλpir or BW20767 was used as a host for molecular cloning experiments. BW20767 is a tra^+ strain that was also used as a donor for conjugations between *E. coli* and *P. stutzeri* strains. Plasmids pAH120 and pLA2 (8) were obtained from Barry Wanner (Purdue University, Lafayette, Ind.).

* Corresponding author. Mailing address: Department of Microbiology, University of Illinois, B103 Chemical and Life Sciences Laboratory, 601 S. Goodwin Ave., Urbana, IL 61801. Phone: (217) 244-1943. Fax: (217) 244-6697. E-mail: metcalf@uiuc.edu.

TABLE 1. Bacterial strains used for this study

Species and strain	Relevant characteristics	Construction or reference
Escherichia coli		
DH5α/λpir	*λpir* φ80*dlacZΔM15* Δ(*lacZYA-argG*)*U169 recA1 hsdr17 deoR thi-1 supE44 gyrA96 relA1*	21
S17-1	RP4-2-Tc::Mu-1 Kan::Tn*7* integrant *recA1 proA creB510 hsdR17 endA1 supE44 thi*	25
BW20767	RP4-2-Tc::Mu-1 Kan::Tn*7* integrant *leu-63*::IS*10 recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (ΔMluI)::*pir*$^+$	18
Pseudomonas stutzeri		
WM567	Spontaneous Str^r mutant of *P. stutzeri* WM536, Hpt$^+$ Pt$^+$	20
WM2033	*ptxE*::*lacZ* Str^r Hpt$^+$ Pt$^+$	Suc^r WM567 segregant of pAW30[a]
WM2757	Δ*ptxE* Str^r Hpt$^+$ Pt$^+$	Suc^r WM567 segregant of pAW28[a]
WM2106	Δ*ptxE*::*lacZ* Str^r Hpt$^+$ Pt$^+$	Suc^r WM567 segregant of pAW29[a]
WM2940	pAW41 integrants Str^r Hpt$^+$ Pt$^+$	Kan^r WM567 integrant of pAW41
WM3021	pAW41 integrants Δ*ptxE* Str^r Hpt$^+$ Pt$^+$	Kan^r WM2757 integrant of pAW41
WM4275	Δ*phoB* Str^r Hpt$^-$ Pt$^-$	Suc^r WM567 segregant of pAW84[a]
WM4296	Δ*phoR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM567 segregant of pAW86[a]
WM4294	Δ*phoBR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM567 segregant of pAW85[a]
WM4268	*ptxE*::*lacZ* Δ*phoB* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2033 segregant of pAW84[a]
WM4261	*ptxE*::*lacZ* Δ*phoR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2033 segregant of pAW86[a]
WM4300	*ptxE*::*lacZ* Δ*phoBR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2033 segregant of pAW85[a]
WM4340	pAW41 integrants Δ*phoB* Str^r Hpt$^-$ Pt$^-$	Kan^r WM4275 integrant of pAW41
WM4341	pAW41 integrants Δ*phoR* Str^r Hpt$^-$ Pt$^-$	Kan^r WM4296 integrant of pAW41
WM4342	pAW41 integrants Δ*phoBR* Str^r Hpt$^-$ Pt$^-$	Kan^r WM4294 integrant of pAW41
WM4269	Δ*ptxE*::*lacZ* Δ*phoB* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2106 segregant of pAW84[a]
WM4292	Δ*ptxE*::*lacZ* Δ*phoR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2106 segregant of pAW86[a]
WM4288	Δ*ptxE*::*lacZ* Δ*phoBR* Str^r Hpt$^-$ Pt$^-$	Suc^r WM2106 segregant of pAW85[a]

[a] Integration and segregation of pAW19-derived plasmids harboring the *sacB* gene were done as described in reference 20.

Media and growth of cultures. The media used throughout were previously reported (32). Tryptone-yeast extract-agar containing an appropriate antibiotic was used for the selection of transformants and exconjugants of strain constructions unless otherwise indicated. A 0.2% glucose–MOPS [3-(*N*-morpholino)propanesulfonic acid] minimal medium was used for the growth of *P. stutzeri* strains on various phosphorus sources and for the screening and selection of proline auxotrophs. Antibiotics were used at the following concentrations for plasmid propagation and strain construction in *E. coli*: kanamycin, 50 μg/ml; streptomycin, 100 μg/ml. For the integration and maintenance of pAW41 in the *P. stutzeri* WM2940 chromosome, kanamycin was used at 10 μg/ml.

Screening for phosphate starvation induction of alkaline phosphatase in *E. coli* was done on 0.2% glucose–MOPS minimal medium containing 0.1 mM P_i and 60 μg of 5-bromo-4-chloro-3-indolyl-phosphate (XP) (Research Products International Corp., Mt. Prospect, Ill.)/ml. Screening for phosphate starvation induction of fusions to the *lacZ* gene, which encodes β-galactosidase, was done on 0.2% glucose–MOPS minimal medium containing 0.1 mM P_i and 32 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Research Products International Corp.)/ml. For reporter gene fusion analysis, *P. stutzeri* strains harboring a *lacZ* reporter gene fusion were grown in glucose-MOPS minimal medium containing either 0.12% glucose and 2 mM P_i (excess P_i) or 1.0% glucose and a 0.1 mM concentration of one of the following phosphorus sources (limiting P_i): P_i, hypophosphite, phosphite, or methylphosphonate. All phosphorus sources were purchased from Sigma (St. Louis, Mo.), and solutions were made immediately prior to use and then filter sterilized. Cultures were harvested at stationary phase (optical density at 600 nm [OD_{600}], ca. 1.0) and cell extracts were made as described below.

DNA methods. Standard methods for the isolation and manipulation of chromosomal and plasmid DNAs were used throughout (3). DNA hybridization reactions were done by using the DIG system (Roche, Mannheim, Germany) according to the manufacturer's instructions. DNA sequencing was performed by using an ABI Prism BigDye Terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, Calif.) per the manufacturer's instructions and were analyzed at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, Urbana.

Identification and cloning of *P. stutzeri phoBR*. The *phoBR* operons and flanking sequences of six pseudomonad species were aligned with ClustalW (28). Highly conserved regions of DNA sequence were used as the basis for degenerate primer design to amplify the *phoBR* operon from the *P. stutzeri* chromosome. The *P. stutzeri phoBR* operon and flanking sequence were amplified by a PCR using Accuzyme DNA polymerase (Bioline USA Inc., Randolph, Mass.) and the following degenerate primers: 5′-AATTYCGTTATCTAATGCG-3′, which anneals to the *P. stutzeri phoBR* region 53 bp upstream of the putative PhoB translational start site, and 5′-CRAGYYGAAGGGTCCATG-3′, which anneals to the *P. stutzeri phoBR* region 115 bp downstream of the translational stop codon of PhoR, resulting in the amplification of a 2,224-bp fragment. The resulting PCR fragment was cloned into the pCR4-TOPO vector by use of a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions, creating plasmid pAW83. The inserted PCR fragment was sequenced initially by using M13 reverse and forward standard primers (Invitrogen) followed by sequencing with sequence-specific internal primers.

Plasmid constructions. Plasmid pAW41 harbors an *htxA*::*lacZ* translational fusion and the *oriT* sequence for plasmid transfer by conjugation in a Kan^r CRIM plasmid (8). In the first step of construction, *oriT* was amplified by the use of *Pfu* Turbo DNA polymerase (Invitrogen) as described previously (11). The resulting PCR fragment was digested with ClaI and inserted into the same sites of pAH120 (8) to create pAW38. In the second step, the 1.0-kbp region upstream of *htxA*, including the *htxA* translational start codon and ribosomal binding site, was amplified by a PCR using the following primers: 5′-GGCGCGCCCATATGGA TGCTCCAAGGTCTTCCAA-3′ and 5′-GGCGCGCCCTGCAGTCTAGAGT GGCTATGTCCTGGGCGTT-3′, which insert NdeI and PstI sites, respectively (restriction sites are underlined). The resulting PCR product was digested with NdeI and PstI and inserted into the same sites of pLA2 (8) to construct a translational *htxA*::*lacZ* fusion. Finally, a BamHI-PstI fragment carrying the *htxA*::*lacZ* translational fusion was inserted into the same sites of pAW38 to create pAW41.

For the construction of *P. stutzeri* strains with a chromosomal *ptxE*::*lacZ* fusion, pAW27 and pAW30 were constructed as derivatives of pAW19. Plasmid pAW19 is a Kan^r derivative of the suicide plasmid pWM91 that can be transferred by conjugation and that carries the *sacB* gene for counterselection of sucrose-resistant plasmid segregants (18). Plasmid pAW30 harbors a *ptxE*::*lacZ* transcriptional fusion and was created by inserting the *lacZ* gene (including its own ribosomal binding site) between the 1.0-kbp sequence directly upstream of, and including, the *ptxE* translational stop codon and the 1.0-kbp sequence directly downstream of the *ptxE* translational stop codon. Both the upstream and downstream sequences were amplified by PCRs using *Taq* DNA polymerase (Invitrogen). Primers 5′-GGCGGCACTAGTACATAGGGTCGGCAGTGCG

C-3′ and 5′-GGCGGCGCGGCCGCTTATCCAGCTAGATCCGCCT-3′, which introduce a SpeI and a NotI site, respectively, were used to amplify the upstream sequence. The 1.0-kbp downstream fragment was amplified with primers 5′-GGCGGCGCGGCCGCGGTGATGGATGGTTGCGATC-3′ and 5′-GGCGGCGAGCTCCAGCGTGGCGTAGAGCTGCG-3′, which incorporate a NotI and a SacI site, respectively. The resulting PCR products were digested with the appropriate restriction enzymes and were inserted into the SpeI and SacI sites of pAW19 in a three-fragment ligation to create pAW27. The *lacZ* gene was amplified from *E. coli* S17-1 genomic DNA with the primers 5′-GGCGGCGCGGCCGCAGGAAACAGCTATGACCATG-3′ and 5′-GGCGGCGCGGCCGCTTATTTTTGACACCAGACCA-3′, which introduce NotI sites immediately upstream of the ribosomal binding site of the *lacZ* gene and immediately downstream of the *lacZ* translational stop codon. The resulting PCR fragment was digested with NotI and inserted into the same sites of pAW27 to create pAW30.

For the construction of *phoB*, *phoR*, and *phoBR* deletion mutants of *P. stutzeri*, plasmids pAW84, pAW85, and pAW86 were constructed from pAW19. Plasmid pAW84 carries ca. 300 bp of 5′ *phoB* and its upstream flanking sequence ligated to ca. 300 bp of 3′ *phoB* and its downstream flanking sequence, resulting in an in-frame 46-amino-acid deletion of the *P. stutzeri phoB* gene. Both the upstream and downstream *phoB* sequences were amplified by PCRs using Platinum *Pfx* polymerase (Invitrogen). Primers 5′-GGATCCACTAGTTAATTTCGTTATCTAATGCC-3′ and 5′-GGATCCGCGGCCGCTGAGCATGATGATCGGCGTGTCG-3′, which incorporate SpeI and NotI sites, respectively, were used to amplify the upstream *phoB* sequence. The downstream *phoB* sequence was amplified with the following primers: 5′-GGATCCGCGGCCGCGGCGGCCTGCTGCTCGATCC-3′ and 5′-GGATCCGAGCTCTCAGCTTTTGCTGGAGAAACG-3′, which incorporate NotI and SstI sites, respectively. The resulting PCR fragments were digested with the appropriate restriction enzymes and were inserted into the SpeI and SstI sites of pAW19 in a three-fragment ligation to create pAW84. Plasmid pAW86 carries a 234-amino-acid in-frame deletion of PhoR and was constructed in a similar manner. Primers 5′-GGATCCACTAGTTTGAATCAGGACTGGCAAGG-3′ and 5′-GGATCCGCGGCCGCGGCGCGATCGATGATGCCTTGC-3′, which incorporate SpeI and NotI sites, respectively, were used to amplify the upstream *phoR* fragment, and primers 5′-GGATCCGCGGCCGCGTACACGCCCGATGGTGGC-3′ and 5′-GGATCCGAGCTCTCAGCGTTCGGACACCTGGC-3′, which incorporate NotI and SstI restriction sites, respectively, were used to amplify the *phoR* downstream fragment. Plasmid pAW85 carries a 1,512-bp internal deletion of the *phoBR* operon in which only the 5′-most 250 bp of *phoB* and the 3′-most 298 bp of *phoR* remain. This plasmid was constructed by inserting the SpeI-NotI upstream *phoB* fragment of pAW84 and the NotI-SstI downstream *phoR* fragment of pAW86 into the SpeI and SstI sites of pAW19 in a three-way ligation.

Genetic techniques. Plasmids pAW30 and pAW41 were introduced into *P. stutzeri* WM567 by conjugation as previously described (11). The desired deletion and reporter gene fusion strains resulting from double recombination events were acquired by *sacB* counterselection as described previously (20).

For the construction of an *htxA*::*lacZ* fusion in *P. stutzeri*, an exconjugant resulting from the integration of pAW41 via homologous recombination at the *htx* promoter region was isolated on glucose-MOPS minimal medium containing 0.1 mM P_i, 10 μg of kanamycin/ml, and X-Gal. This strain carries both the *htxA*::*lacZ* translational fusion and an intact *htx* operon. Correct construction of the chromosomal deletions and reporter gene fusions in *P. stutzeri* was verified by DNA hybridization analysis (data not shown).

RT-PCR. Total RNAs were isolated from cultures of *P. stutzeri* WM88 grown to mid-logarithmic phase (OD_{600}, ca. 0.6) in 0.2% glucose–MOPS minimal medium with 0.5 mM hypophosphite as the sole source of phosphorus. RNAs were isolated with an RNeasy mini kit containing an RNAprotect bacterial reagent (Qiagen Inc., Valencia, Calif.) per the manufacturer's instructions. For the removal of contaminating chromosomal DNA, the RNA preparation was digested with amplification-grade DNase I (Invitrogen). DNase I-treated RNA was then used as a template in a reverse transcription (RT) assay by 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 by using Platinum *Pfx* DNA polymerase (Invitrogen) per the manufacturer's instructions. Both a positive control, in which only chromosomal DNA was added to the PCR, and a negative control, in which only RNA without the RT step was used in the PCR, were run under identical PCR amplification conditions. The primers used to amplify each *ptx* junction sequence are listed in Table 2.

Enzymatic assays. β-Galactosidase specific activities were determined by continuous assaying in 1-ml volumes and are reported in standard units (micromoles per minute per milligram). Extracts were made from *P. stutzeri* cultures grown as described above. Cells were harvested by centrifugation and the entire cell pellet was resuspended in 50 mM Tris-Cl, pH 8.0. The cells were lysed by sonication with two 30-s pulses at 4°C or by passage through a French press at 13,000 lb/in^2. The resulting crude cell extract was centrifuged at 15,000 × *g* for 20 min and the supernatant was removed for activity assays. β-Galactosidase assays were carried out in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, with 2.7 mM *o*-nitrophenyl-β-D-galactoside (ONPG) (Sigma) as a substrate. The release of *o*-nitrophenol was monitored as an increase in the absorbance at 420 nm, and an extinction coefficient of 4,112 M^{-1} cm^{-1} was used to calculate *o*-nitrophenol production. Protein concentrations were determined by using the Coomassie Plus protein assay reagent (Pierce, Rockford, Ill.) as recommended.

Nucleotide sequence accession numbers. The GenBank accession number for the *P. stutzeri* WM88 *phoBR* DNA sequence determined for this study is AY590886.

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

Amplified junction	Primer set (5′–3′)	Predicted product size (bp)
ptxAB	ATGAGCCGGTAGCCAGTCT, AAATACGCCAGGTCGATACG	561
ptxBC	GGGCAGGACTACGAACAACA, TCGATAGCCCGAAAAGTCTG	611
ptxCD	CATGGTCGGCAAGTTCTTC, CCGACTACACGCAGCTCA	563
ptxDE	GAGCTGCTTGCCCTCGTA, CCATGCAGGGCTTCTAGC	598

RESULTS

The genes within the *ptx* locus form a transcriptional unit. All of the open reading frames in the *ptx* locus either overlap one another or are separated by at most nine bases. This suggests that the *ptxABCDE* genes form an operon, but this had not been experimentally verified. We determined that the *ptx* genes are cotranscribed by performing RT-PCRs with the junction sequences between each of the genes (Fig. 1). Primers were designed to amplify ca. 300 bp upstream and downstream of the intergenic regions of each gene to yield amplification

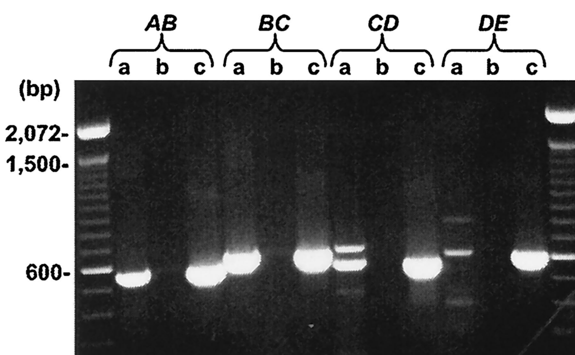

FIG. 1. RT-PCRs with total RNAs prepared from *P. stutzeri* WM567 grown on hypophosphite as the sole P source to determine the operon structure of *ptx*. Lanes a, complete RT reactions; lanes b, negative controls for which no reverse transcriptase was added to the reaction; lanes c, PCR-positive controls in which chromosomal DNA was used as the template. The left- and rightmost lanes contain a 100-bp ladder. The junction sequences amplified are indicated above the reactions. For a list of primers used and the predicted PCR product size for each reaction, refer to Table 2.

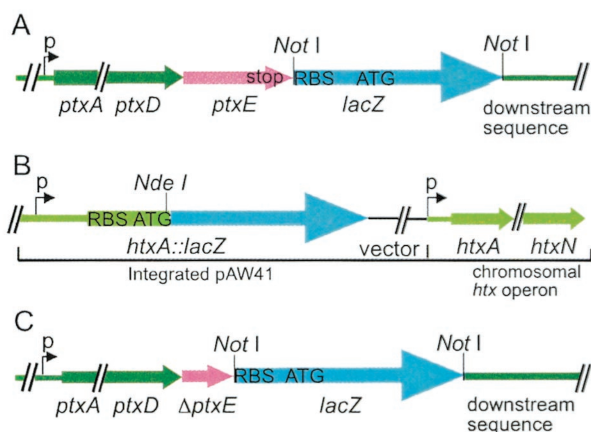

FIG. 2. Structures of chromosomal reporter gene fusions in *P. stutzeri*. (A) Structure of *ptxE*::*lacZ* transcriptional fusion. (B) Structure of *htxA*::*lacZ* translational fusion, showing the integrant structure formed by integration of pAW41 at the *htx* promoter region and the native promoter. (C) Structure of Δ*ptxE*::*lacZ* transcriptional fusion. The diagram was not drawn to scale.

products of ca. 600 bp. Such products would be obtained only if the mRNAs spanned the junction of the two genes, indicating that they were cotranscribed. Although several additional bands were present in some of the reactions due to nonspecific amplification, a significant PCR product corresponding to the predicted size was amplified, supporting the conclusion that the *ptx* genes form an operon. With the same method, the genes in the *htx* locus were also determined to be cotranscribed (34a).

Expression of *htx* and *ptx* operons is induced under phosphate starvation conditions. To examine the regulation of expression of the *ptx* operon in *P. stutzeri*, we constructed a chromosomal *ptxE*::*lacZ* transcriptional fusion (Fig. 2A) (strain WM2033) and used it to measure *ptx* gene expression. WM2033 was grown to stationary phase in triplicate cultures with different phosphorus sources and with either excess or limiting P_i, and β-galactosidase activities in the cell extracts were measured (Table 3). To verify that the cultures were starved for P_i, we also measured the activity of native phosphatase in each of the extracts, as the expression of phosphatase is only induced upon P_i starvation (S. E. Neuhaus, A. K. White, and W. W. Metcalf, unpublished data). Expression of the *ptx* operon was induced 14-fold during growth on limiting P_i and up to 22-fold during growth on phosphite relative to the expression levels on excess P_i. Thus, expression of the *ptx* operon is induced by P_i starvation. Similar expression levels were observed for a *ptxA*::*lacZ* translational fusion in *E. coli* in response to P_i starvation (data not shown).

Expression analysis of the *htx* operon was done in a similar manner. Due to the large size of the *htx* operon (11.8 kbp), a reporter gene fusion was constructed to measure expression levels at the *htx* promoter rather than at the distal end of the operon. A chromosomal *htxA*::*lacZ* translational fusion was constructed by integration, via homologous recombination at the *htx* promoter, of a suicide plasmid (pAW41) carrying a 1.0-kbp region directly upstream of the translational start site of *htxA* (Fig. 2B) (strain WM2940). This allowed for measurements of the expression levels at the plasmid-borne *htx* promoter without disrupting expression at the native promoter. Strain WM2940 was grown under the conditions described above and its β-galactosidase activity was measured (Table 3). Compared to the expression level during growth on 2 mM P_i (excess P_i), an 11-fold induction of *htx* expression in response to phosphate starvation (0.1 mM P_i) was observed. The induction of expression was slightly higher for growth on phosphite or hypophosphite as the phosphorus source, resulting in a 13- and 17-fold induction, respectively.

To determine if the presence of the reduced phosphorus compounds that act as phosphorus substrates for *P. stutzeri* could specifically induce the expression of either the *htx* or *ptx* operon in the presence of P_i, we grew the reporter gene fusion strains on 2 mM P_i in addition to 0.1 mM phosphite, hypophosphite, or the organic reduced phosphorus compound methylphosphonate. No induction of expression of either the *ptx* or *htx* operon was observed (Table 3).

Identification of *P. stutzeri phoBR*. The induction of the *htx* and *ptx* operons in response to P_i starvation suggested that *htx* and *ptx* might be regulated in a *phoBR*-dependent manner.

TABLE 3. Expression of a *ptxE*::*lacZ* transcriptional fusion and an *htxA*::*lacZ* translational fusion in *P. stutzeri* in response to growth on different P sources

P source[c] (concn [mM])	*htxA*::*lacZ* expression[a]		*ptxE*::*lacZ* expression[b]	
	β-Galactosidase activity[d]	Fold induction[e]	β-Galactosidase activity[d]	Fold induction[e]
P_i (2)	0.17 ± 0.05	1	0.01 ± 0.00	1
P_i (0.1)	1.80 ± 0.77	10.6	0.15 ± 0.02	15
Pt (0.1)	2.25 ± 0.95	13.2	0.20 ± 0.03	20
Hpt (0.1)	2.98 ± 0.64	17.5	0.17 ± 0.02	17
P_i (2) + Pt (0.1)	0.18 ± 0.07	1.1	0.01 ± 0.00	1
P_i (2) + Hpt (0.1)	0.19 ± 0.06	1.1	0.01 ± 0.00	1
P_i (2) + Mpn (0.1)	0.13 ± 0.02	NA[g]	ND[f]	NA[g]

[a] *P. stutzeri* WM2940.
[b] *P. stutzeri* WM2033.
[c] The P sources were added to either 2 mM (excess P with a limiting carbon source) or 0.1 mM (P starvation with excess carbon source). P_i, Pt, Hpt, and Mpn are abbreviations for phosphate, phosphite, hypophosphite, and methylphosphate, respectively.
[d] β-Galactosidase activities were determined from duplicate assays of triplicate cultures and are reported in standard units as means ± standard deviations.
[e] Induction relative to the expression observed with growth on 2 mM P_i.
[f] ND, Not detected. The limit of detection was 0.005 U.
[g] NA, Not applicable.

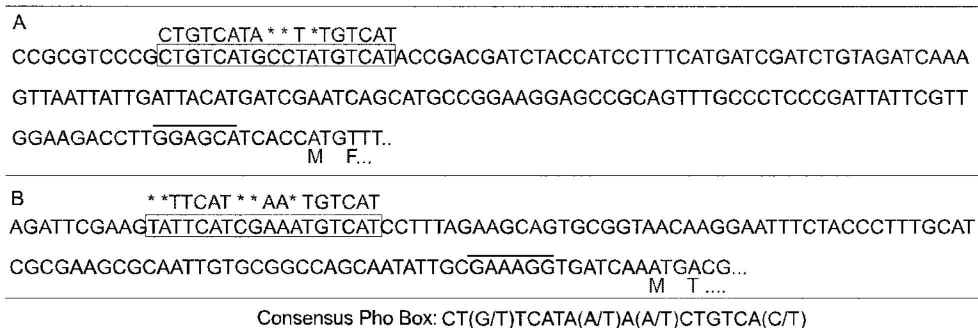

FIG. 3. DNA sequences of promoter regions of *htx* operon (A) and *ptx* operon (B). The partial deduced amino acid sequence of each protein is shown below the coding sequence. The boxed sequence represents a putative Pho box and the match to the consensus sequence is shown above it. The predicted ribosomal binding site for each sequence is indicated by a line above the sequence.

This possibility was further supported by the presence of well-conserved putative Pho boxes located within the promoter regions of the *htx* and *ptx* operons (Fig. 3). Although the presence of *phoBR* homologs in *P. stutzeri* had not been determined previously, homologs of these genes have been identified in the published genome sequences of several pseudomonad species. To examine PhoBR-dependent regulation of the *htx* and *ptx* operons in the native host, we identified *P. stutzeri phoBR* as follows. The *phoBR* and flanking sequences of six pseudomonads were aligned with ClustalW (28), and degenerate primers were designed from conserved sequences just upstream and downstream of the *phoBR* operons of these organisms. Using these primers, we amplified a ca. 2.2-kbp PCR fragment, consistent with the predicted size of the *phoBR* operons from other pseudomonads. The fragment was cloned, and sequence analysis indicated the presence of two open reading frames arranged in a putative operon. Comparisons of the predicted amino acid sequences encoded by the two open reading frames to those in the UniProt database indicated that the first open reading frame, of 690 bp, encodes a protein of 229 amino acids that is 90 to 93% identical on the amino acid level to the PhoB proteins of other pseudomonads and 42% identical to *E. coli* PhoB. The second open reading frame (located 68 bp downstream of the stop codon of *phoB*) is 1,299 bp long and encodes a protein of 433 amino acids that shares 69 to 72% amino acid sequence identity with the PhoR proteins from other pseudomonads and 42% identity with the PhoR protein of *E. coli*. Thus, based on sequence analysis, the cloned fragment encodes a *phoBR* operon of 2,060 bp from *P. stutzeri*, in addition to 49 bp directly upstream of the PhoB translational start site and 115 bp directly downstream of the PhoR translational stop codon.

Hypophosphite and phosphite oxidation is *phoBR* dependent in *P. stutzeri*. To examine the role of *phoBR* on the utilization of the reduced phosphorus compounds hypophosphite and phosphite in *P. stutzeri*, we constructed in-frame deletions in either *phoB* alone (Δ*phoB*), *phoR* alone (Δ*phoR*), or both (Δ*phoBR*). To examine the phenotypes of these mutants with respect to the oxidation of reduced phosphorus compounds, we streaked the mutants alongside the wild-type parental strain on glucose-MOPS minimal medium containing one of a variety of phosphorus sources, as described above. The absence of growth on any of these substrates by any of the *phoBR* mutants contrasted with the robust growth observed for the wild-type strain (WM567) and demonstrated that both hypophosphite and phosphite oxidation is dependent on functional *phoBR* (data not shown). Similarly, growth on methylphosphonate, a substrate for the two C-P lyase pathways encoded by *htxBCDEFGHIJKLMN* and *phnC-phnP*, which are predicted to be Psi operons, was also abolished in the *phoBR* mutants (data not shown).

Several other interesting phenotypes were observed for the *phoBR* mutants. A marked decrease in growth on low-P_i solid medium was observed for the Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutants compared to that of the wild-type strain WM567. This phenotype was not observed for medium with excess P_i. To examine the nature of the growth defect in the mutant strains, we performed a growth analysis of the wild type and the mutants in broth cultures. Although the doubling times for the wild type and the mutants on low-P_i medium were similar (ca. 2.4 h), the maximum OD_{600} reached by the wild-type strain was 0.74 ± 0.02, whereas the maximum OD_{600} reached by the mutant strains was only 0.33 ± 0.01. This indicates that the decrease in growth observed for the *phoBR* mutants was due to a decrease in maximum growth yield rather than to an increase in the doubling time.

Expression of *htx* and *ptx* operons is *phoBR* dependent in *P. stutzeri*. To examine the mechanism of *phoBR* regulation of the *htx* and *ptx* operons in *P. stutzeri*, we compared the expression of the *ptxE*::*lacZ* and *htxA*::*lacZ* fusions in the wild type and the Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutants of *P. stutzeri*. The appropriate strains were grown under P_i starvation and P_i excess conditions and the β-galactosidase activities were measured as described above. Both the *ptx* and *htx* induction levels in the wild-type strains (WM2033 and WM2940, respectively) with low P_i and high P_i were similar to those that were previously observed (Table 3). However, the induction of expression of both the *ptx* and *htx* fusions in response to P_i starvation was completely lost in each of the mutants (Table 4). Similar decreases in response to P_i starvation were observed for each of the mutants, indicating that the effects of a null mutation in *phoB*, *phoR*, or *phoBR* are the same. These data provide additional support for a difference in the regulation of the Pho regulons of *E. coli* and *P. stutzeri*, as the constitutive expression of *ptx* or *htx* was not observed for the Δ*phoR* mutant. Thus, P_i starvation-dependent expression of the *htx* and *ptx* operons in

TABLE 4. Expression of the *ptx* and *htx* operons in wild-type and Δ*phoB*, Δ*phoR*, and Δ*phoBR P. stutzeri* strains

Strain[a]	β-Galactosidase activity[b]		Fold induction[c]
	0.1 mM P_i	2 mM P_i	
ptx expression			
ptxE::*lacZ*	0.22 ± 0.02	0.01 ± 0.00	16
ptxE::*lacZ* Δ*phoB*	ND	0.02 ± 0.00	NA
ptxE::*lacZ* Δ*phoR*	ND	0.02 ± 0.00	NA
ptxE::*lacZ* Δ*phoBR*	ND	0.02 ± 0.01	NA
htx expression			
htxA::*lacZ*	1.43 ± 0.14	0.08 ± 0.01	18
htxA::*lacZ* Δ*phoB*	0.09 ± 0.02	0.08 ± 0.01	1
htxA::*lacZ* Δ*phoR*	0.06 ± 0.00	0.07 ± 0.01	<1
htxA::*lacZ* Δ*phoBR*	0.08 ± 0.00	0.09 ± 0.01	<1
ptx expression in Δ*ptxE* strain			
Δ*ptxE*::*lacZ*	0.20 ± 0.04	0.01 ± 0.00	20
Δ*ptxE*::*lacZ* Δ*phoB*	ND	ND	NA
Δ*ptxE*::*lacZ* Δ*phoR*	ND	ND	NA
Δ*ptxE*::*lacZ* Δ*phoBR*	ND	0.02 ± 0.00	NA

[a] The strains used were *ptxE*::*lacZ* (WM2033), *ptxE*::*lacZ* Δ*phoB* (WM4268), *ptxE*::*lacZ* Δ*phoR* (WM4261), *ptxE*::*lacZ* Δ*phoBR* (WM4300), *htxA*::*lacZ* (WM2940), *htxA*::*lacZ* Δ*phoB* (WM4340), *htxA*::*lacZ* Δ*phoR* (WM4341), *htxA*::*lacZ* Δ*phoBR* (WM4342), Δ*ptxE*::*lacZ* (WM2106), Δ*ptxE*::*lacZ* Δ*phoB* (WM4269), Δ*ptxE*::*lacZ* Δ*phoR* (WM4292), and Δ*ptxE*::*lacZ* Δ*phoBR* (WM4288).

[b] β-Galactosidase activities were determined from duplicate assays of triplicate cultures and are reported in standard units as means ± standard deviations. ND, not detected. The detection limit was 0.01 U.

[c] Induction relative to the expression observed with growth on 2 mM P_i. NA, not applicable.

P. stutzeri is dependent on *phoBR*, and the regulation of these operons occurs at the level of transcription.

***ptxE* does not play a role in the regulation of the *htx* or *ptx* operon in response to P_i starvation.** The *ptxE* gene encodes a putative transcriptional regulator in the LysR family, suggesting that it might be involved in regulating the expression of the *ptx* and *htx* operons. To examine the role of *ptxE*, we constructed a chromosomal *ptxE* internal deletion mutant in both the *ptxE*::*lacZ* (WM2106) (Fig. 2C) and *htxA*::*lacZ* (strain WM3021) fusion backgrounds. Surprisingly, there was no significant change in expression level for either the *ptx* or *htx* operon in the Δ*ptxE* strain compared to the wild type after growth on each phosphorus source (data not shown). To determine if a role for *ptxE* could be observed in the absence of *phoBR*, we constructed Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutations in the Δ*ptxE*::*lacZ* fusion background. However, again Δ*ptxE* had no effect on the induction patterns in response to P_i starvation (Table 4). Thus, the role for *ptxE* in the expression of *htx* and *ptx* remains unclear.

DISCUSSION

Our expression analysis of *ptxE*::*lacZ* and *htxA*::*lacZ* fusions in *P. stutzeri* clearly demonstrated that both the *htx* and *ptx* genes are regulated in response to P_i starvation. Furthermore, an analysis of the *htx* and *ptx* reporter gene fusions in the wild type compared to those in Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutants of *P. stutzeri* confirmed that the regulation of the *htx* and *ptx* operons is *phoBR* dependent. Therefore, the *htx* and *ptx* operons, encoding products for the oxidation of the inorganic reduced P compounds hypophosphite and phosphite, are novel members of the Pho regulon of *P. stutzeri*, thus providing convincing evidence that the physiological role of these genes is P_i acquisition from an alternate phosphorus source.

A growth defect was observed for the Δ*phoB*, Δ*phoR*, and Δ*phoBR* mutants of *P. stutzeri* on 0.1 mM P_i compared to the growth of the wild type. The mutant phenotype appeared to be due to a decrease in the maximum growth yield rather than to an increase in doubling time. The inability of the mutants to continue growing suggests that a high-affinity P_i transport system required for growth on low levels of P_i is no longer expressed in the absence of PhoBR. Although nothing is known about P_i transport in *P. stutzeri*, PhoBR-dependent high-affinity P_i transport systems have been characterized for numerous bacteria, including *E. coli* and several pseudomonads (23, 35, 37). It is reasonable to suspect that *P. stutzeri* also possesses such a transport system as part of its Pho regulon that would be required for growth on limiting P_i.

The sequence similarity between *ptxE* and other regulatory proteins of the LysR family (32% amino acid sequence identity to CbbR of *Rhizobium meliloti*), in addition to the presence of a conserved helix-turn-helix motif for DNA binding (9), suggests that PtxE might act as a regulator of the *htx* or *ptx* genes. Despite these properties, PtxE has no apparent role in the regulation of the *htx* or *ptx* genes in response to P_i starvation, as seen by the absence of a measurable effect on the expression levels of these genes in the wild-type and Δ*ptxE* strains in the presence or absence of *phoBR*. Perhaps this observation should not be surprising considering that no genes of the Pho regulon have yet been found to be under individual regulatory control in addition to the regulatory effects exerted by *phoBR* (30).

The data presented in this report, in addition to the large numbers of bacterial species reported to grow on hypophosphite and phosphite as sole sources of phosphorus, provide strong evidence for both the presence of these reduced phosphorus compounds in the environment and the significant role that they play as alternate phosphorus sources for environmental organisms.

ACKNOWLEDGMENTS

We are grateful to Barry Wanner for generously providing strains and plasmids and to Marlena Wilson, Adam Guss, and Shannon Neuhaus for their efforts in transposon mutagenesis.

This work was supported by grant GM59334 from the National Institute of General Medical Sciences.

REFERENCES

1. **Adams, F., and J. P. Conrad.** 1953. Transition of phosphite to phosphate in soils. Soil Sci. **75:**361–371.
2. **Argast, M., and W. Boos.** 1980. Coregulation in *Escherichia coli* of a novel transport system for *sn*-glycerol-3-phosphate and outer membrane protein Ic (e, E) with alkaline phosphatase and phosphate-binding protein. J. Bacteriol. **143:**142–150.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Current protocols in molecular biology, vol. 1 and 2. John Wiley & Sons, New York, N.Y.
4. **Brunner, U., T. G. Chasteen, P. Ferloni, and R. Bachofen.** 1995. Chromatographic determination of phosphine (PH_3) and hydrogen sulfide (H_2S) in the headspace of anaerobic bacterial enrichments using flame photometric detection. Chromatographia **40:**399–403.
5. **Costas, A. M., A. K. White, and W. W. Metcalf.** 2001. Purification and characterization of a novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88. J. Biol. Chem. **276:**17429–17436.
6. **Foster, T. L., L. Winans, Jr., and S. J. Helms.** 1978. Anaerobic utilization of

phosphite and hypophosphite by *Bacillus* sp. Appl. Environ. Microbiol. **35:** 937–944.

7. **Gassman, G., and F. Schorn.** 1993. Phosphine from harbor surface sediments. Naturwissenschaften **80:**78–80.
8. **Haldimann, A., and B. L. Wanner.** 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J. Bacteriol. **183:**6384–6393.
9. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
10. **Imazu, K.** 1998. Enhanced utilization of phosphonate and phosphite by *Klebsiella aerogenes*. Appl. Environ. Microbiol. **64:**3754–3758.
11. **Larsen, R. A., M. M. Wilson, A. M. Guss, and W. W. Metcalf.** 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch. Microbiol. **178:**193–201.
12. **Lauwers, A. M., and W. Heinen.** 1977. Alterations of alkaline phosphatase activity during adaptation of *Escherichia coli* to phosphite and hypophosphite. Arch. Microbiol. **112:**103–107.
13. **Makino, K.** 1986. Nucleotide sequence of the *phoB* gene, the positive regulatory gene for the phosphate regulon of *Escherichia coli* K-12. J. Mol. Biol. **190:**37–44.
14. **Makino, K., H. Shinagawa, M. Amemura, T. Kawamoto, M. Yamada, and A. Nakata.** 1989. Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. J. Mol. Biol. **210:**551–559.
15. **Makino, K., H. Shinagawa, M. Amemura, S. Kimura, A. Nakata, and A. Ishihama.** 1988. Regulation of the phosphate regulon of *Escherichia coli.* Activation of *pstS* transcription by PhoB protein in vitro. J. Mol. Biol. **203:**85–95.
16. **Makino, K., H. Shinagawa, M. Amemura, and A. Nakata.** 1986. Nucleotide sequence of the *phoR* gene, a regulatory gene for the phosphate regulon of *Escherichia coli*. J. Mol. Biol. **192:**549–556.
17. **Malacinski, G., and W. A. Konetzka.** 1966. Bacterial oxidation of orthophosphite. J. Bacteriol. **91:**578–582.
18. **Metcalf, W. W., W. Jiang, L. L. Daniels, S. K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZ* alpha for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1–13.
19. **Metcalf, W. W., and B. L. Wanner.** 1991. Involvement of the *Escherichia coli phn* (*psiD*) gene cluster in assimilation of phosphorus in the form of phosphonates, phosphite, Pi esters, and Pi. J. Bacteriol. **173:**587–600.
20. **Metcalf, W. W., and R. S. Wolfe.** 1998. Molecular genetic analysis of phosphite and hypophosphite oxidation by *Pseudomonas stutzeri* WM88. J. Bacteriol. **180:**5547–5558.
21. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
22. **Morton, S. C., D. Glindemann, and M. A. Edwards.** 2003. Phosphates, phosphites, and phosphides in environmental samples. Environ. Sci. Technol. **37:**1169–1174.
23. **Nikata, T., Y. Sakai, K. Shibat, J. Kato, A. Kuroda, and H. Ohtake.** 1996. Molecular analysis of the phosphate-specific transport (*pst*) operon of *Pseudomonas aeruginosa*. Mol. Gen. Genet. **250:**692–698.
24. **Qi, Y., Y. Kobayashi, and F. M. Hulett.** 1997. The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the *pho* regulon. J. Bacteriol. **179:**2534–2539.
25. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
26. **Steed, P. M., and B. L. Wanner.** 1993. Use of the rep technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. J. Bacteriol. **175:**6797–6809.
27. **Surin, B. P., H. Rosenberg, and G. B. Cox.** 1985. Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J. Bacteriol. **161:**189–198.
28. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
29. **Tsubota, G.** 1959. Phosphate reduction in the paddy field I. Soil Plant Food **5:**10–15.
30. **Wanner, B. L.** 1993. Gene regulation by phosphate in enteric bacteria. J. Cell Biochem. **51:**47–54.
31. **Wanner, B. L.** 1992. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. **174:** 2053–2058.
32. **Wanner, B. L.** 1986. Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Mol. Biol. **191:**39–58.
33. **Wanner, B. L., and J. A. Boline.** 1990. Mapping and molecular cloning of the *phn* (*psiD*) locus for phosphonate utilization in *Escherichia coli*. J. Bacteriol. **172:**1186–1196.
34. **White, A. K., and W. W. Metcalf.** 2002. Isolation and biochemical characterization of hypophosphite/2-oxoglutarate dioxygenase. A novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88. J. Biol. Chem. **277:** 38262–38271.

34a. **White, A. K., and W. W. Metcalf.** 2004. Two C-P Lyase Operons in *Pseudomonas stutzeri* and their roles in the oxidation of phosphonates, phosphite, and hypophosphite. J. Bacteriol. **186:**4730–4739.

35. **Willsky, G. R., and M. H. Malamy.** 1980. Characterization of two genetically separable inorganic phosphate transport systems in *Escherichia coli*. J. Bacteriol. **144:**356–365.
36. **Willsky, G. R., and M. H. Malamy.** 1976. Control of the synthesis of alkaline phosphatase and the phosphate-binding protein in *Escherichia coli*. J. Bacteriol. **127:**595–609.
37. **Wu, H., H. Kosaka, J. Kato, A. Kuroda, T. Ikeda, N. Takiguchi, and H. Ohtake.** 1999. Cloning and characterization of *Pseudomonas putida* genes encoding the phosphate-specific transport system. J. Biosci. Bioeng. **87:**273–279.
38. **Yakovleva, G. M., S. K. Kim, and B. L. Wanner.** 1998. Phosphate-independent expression of the carbon-phosphorus lyase activity of *Escherichia coli*. Appl. Microbiol. Biotechnol. **49:**573–578.