The Chemolithoautotroph *Acidithiobacillus ferrooxidans* Can Survive under Phosphate-Limiting Conditions by Expressing a C-P Lyase Operon That Allows It To Grow on Phosphonates▽†

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The chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* is of great importance in biomining operations. During the bioleaching of ores, microorganisms are subjected to a variety of environmental stresses and to the limitations of some nutrients, such as inorganic phosphate (P_i), which is an essential component for all living cells. Although the primary source of phosphorus for microorganisms is P_i, some bacteria are also able to metabolize P_i esters (with a C-O-P bond) and phosphonates (with a very inert C-P bond). By using bioinformatic analysis of genomic sequences of the type strain of *A. ferrooxidans* (ATCC 23270), we found that as part of a Pho regulon, this bacterium has a complete gene cluster encoding C-P lyase, which is the main bacterial enzyme involved in phosphonate (Pn) degradation in other microorganisms. *A. ferrooxidans* was able to grow in the presence of methyl-Pn or ethyl-Pn as an alternative phosphorus source. Under these growth conditions, a great reduction in inorganic polyphosphate (polyP) levels was seen compared with the level for cells grown in the presence of P_i. By means of reverse transcription-PCR (RT-PCR), DNA macroarrays, and real-time RT-PCR experiments, it was found that *A. ferrooxidans phn* genes were cotranscribed and their expression was induced when the microorganism was grown in methyl-Pn as the only phosphorus source. This is the first report of phosphonate utilization in a chemolithoautotrophic microorganism. The existence of a functional C-P lyase system is a clear advantage for the survival under P_i limitation, a condition that may greatly affect the bioleaching of ores.

Phosphonates (Pn) are a class of organic compounds possessing a chemically inert C-P bond that makes them very recalcitrant to microbial degradation. Of the wide variety of Pn present in the environment, some, such as 2-aminoethyl-Pn and phosphonomycin, have biogenic origins (25). Many other Pn are derived from chemical synthesis and have extensive industrial uses as detergents, coolant additives, drugs, adhesives, and herbicides, such as glyphosate (*N*-phosphonomethylglycine). The significant amounts of Pn that are released every year have become an environmental problem due to their long residence times and their potential toxicities to some organisms (12).

Genetic capabilities to degrade Pn are present in gram-negative and some gram-positive bacteria. Four classes of enzymes involved in Pn metabolism have been characterized: phosphonoacetate hydrolase, which cleaves specifically phosphonoacetate (17); phosphonopyruvate hydrolase (27); phosphonatase, which cleaves phosphonoacetaldehyde, the transamination product of 2-aminoethyl phosphonic acid (14); and C-P lyase, which is the most widespread enzymatic pathway for Pn degradation, cleaving the C-P bond of a broad spectrum of Pn (32). In *Escherichia coli*, all the functions for phosphonate transport and degradation are encoded by an operon (composed of 14 genes and named *phnCDEFGHIJKLMNOP*) whose expression is induced by phosphate starvation as part of the Pho regulon (34). In this bacterium, C-P lyase is a multienzyme complex encoded by seven genes, *phnGHI JKLM*, and enzymatic activity has been detected only in whole permeabilized cells; it has never been detected in cell extracts (18, 32).

Acidithiobacillus ferrooxidans is a chemolithoautotrophic acidophilic bacterium that obtains its energy from the oxidation of hydrogen, ferrous iron, elemental sulfur, or partially oxidized sulfur compounds (23, 30). This ability makes it of great industrial importance due to its applications in biomining. During these industrial processes, microorganisms are normally subjected to stressing circumstances in their environment, such as temperature and pH changes, nutrient starvation, and the presence of toxic heavy metals, which can affect their physiological conditions. Phosphorus plays an essential part of cell structure and metabolism, forming part of nucleic acids, phospholipids, lipopolysaccharides, nucleotide cofactors, and some proteins, where it is incorporated through posttranslational modification (28). The lack of phosphate (P_i) may therefore greatly affect the bioleaching of minerals (21). Bacteria meet their phosphorus requirements through the assimilation of P_i and the accumulation of various phosphorus containing compounds, such as polyphosphates (polyP) (13). However, Pn can also be used as an alternative phosphorus source when the microorganisms possess the genetic capabilities to transport and degrade them (12, 25, 34).

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† Dedicated to the memory of Arthur Kornberg, a great scientist and friend who introduced us to the "polyP world."

We have previously studied the P_i starvation response (24) and the metabolism of inorganic polyP in *A. ferrooxidans* (2). By using two-dimensional polyacrylamide gel electrophoresis, we isolated a PstS protein which was induced in cultures starved of P_i and found in this acidophilic bacterium orthologs to the *E. coli* Pho regulon genes (31). Further analysis revealed the presence of a complete gene cluster with high identity to the C-P lyase genes from *E. coli*, suggesting that *A. ferrooxidans* may also have the ability to degrade Pn and use them as valuable phosphorus sources.

In this work, we found that *A. ferrooxidans* was able to grow on methyl-phosphonate (M-Pn) or ethyl-phosphonate (E-Pn) as the only phosphorus source. Furthermore, the increased expression levels of C-P lyase genes when the microorganism was grown on M-Pn indicate that *A. ferrooxidans* may utilize the environmental Pn pool, a capacity that confers it a growth and survival advantage relative to other microorganisms unable to utilize Pn in their natural environments. This advantage, in turn, may be important in industrial biomining operations.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The type strain of *A. ferrooxidans* (ATCC 23270) was grown in DSMZ 71 medium with thiosulfate (containing 22 mM P_i) as described before (20) or in ferrous sulfate containing 9K medium (0.17 mM P_i). In spite of the different P_i concentrations, both media had an excess of P_i for the growth of the microorganisms and they were therefore considered a "presence of P_i condition." In control cultures, the P_i salt was not included in the corresponding media. For growth in M-Pn or E-Pn, the P_i salt in each culture medium was replaced with 1 mM concentration of the corresponding phosphonate. Growth was monitored by determining cell numbers under a phase-contrast Olympus BX50 microscope with a Petroff-Hauser counting chamber. Total RNA was extracted when cells reached the late exponential phase of growth.

PolyP quantification. PolyP was quantified by using a two-step conversion into ATP by recombinant *E. coli* polyP kinase (PPK) and quantification of the ATP formed by luciferase to generate light (3). First, polyP was extracted from cell extracts by using glass milk and then it was assayed by using the reverse reaction of PPK in ADP excess. Finally, the ATP content was assayed by using the firefly luciferase ATP assay and the luminescence was measured by using a luminometer (BioScan Lumi/96). Concentration of polyP is given in terms of P_i residues.

RNA techniques. Total RNA was prepared from *A. ferrooxidans* cultures by a hot-phenol method (8), with some modifications. The RNA was subsequently extracted twice with chloroform and precipitated overnight at −80°C. RNA pellets were washed twice with 70% ethanol and resuspended in nuclease-free water. DNA was eliminated by the addition of 30 U RNase-free DNase (Amersham). RNAs were stored at −80°C. For the cotranscription experiments, a reverse primer hybridizing to the *phnM* gene was used and the cDNA synthesis was carried out with 1 μg of total RNA from a culture grown with M-Pn. PCR amplifications were performed with 1 μl of a 1/20 dilution of the cDNA and 25 pmol of each primer. Amplification conditions included an initial 3-min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C and finished by 3 min at 72°C. All primers used are shown in Table 1.

DNA macroarray experiments. The spotting of DNA onto nylon membranes and the hybridization of radiolabeled cDNA probes were carried out as described previously (1, 8). Radiolabeled cDNAs were synthesized with 4 μg of total RNA from cultures grown in M-Pn or in the presence of P_i by using the ImPromII RT system (Promega), a mixture of specific oligonucleotides for the open reading frames (ORFs) of interest and [α-^{32}P]dCTP (Amersham Biosciences). In vitro-transcribed, "spiked" RNA was added to the labeling reaction for normalization purposes (1, 8). After the RT reaction, the RNA template was degraded by alkaline treatment and the cDNA probes were purified by using MicroSpin S-200 columns (Amersham Pharmacia Biotech). After hybridization and washing, the membranes were air dried and exposed to PhosphorImager screens for variable times. The screens were then scanned on a PhosphorImager (Molecular Imager FX system; Bio-Rad) at a resolution of 50 μm/pixel. Analysis and quantification of the spot signals were performed with VersArray 1.0 software (Bio-Rad).

TABLE 1. Oligonucleotides used in this work

Primer	Oligonucleotide sequence	Use(s)[a]
Af-pho84L-N	5′ ATGCTTCAGTGCCGATGC 3′	a
Af-pho84L-R1	5′ AGCACCAACCCAACGACT 3′	a,b
Af-pho84L-D1	5′ GGAAGTTTCAGAAGTGGC 3′	d
Af-phnF-N	5′ CACCAAAGTACCTTACGC 3′	a
Af-phnF-R1	5′ CGGGATCGAATTTATCTATGG 3′	a,b
Af-phnF-D1	5′ CAACGGTGATCTATCCTG 3′	d
Af-phnG-N	5′ GAAATTCGATGAAGCGCC 3′	a
Af-phnG-R1	5′ TCATGACCGCATGGTTTC 3′	a,b
Af-phnG-D1	5′ CCCGCGAAACGCAGATAA 3′	d
Af-phnH-N	5′ TGATGGCATTGGATTGCC 3′	a
Af-phnH-R1	5′ TCAGGCCTCCTCTATTTC 3′	a,b
Af-phnH-D1	5′ TTTTCTCTGCCTGCCGAG 3′	d
Af-phnI-N	5′ ATGTCCTACGTGGCAGTC 3′	a
Af-phnI-R1	5′ TAAGCGTTGCGCACGAGT 3′	a,b
Af-phnI-D1	5′ ATCTGAAACTCCCGCACT 3′	d
Af-phnJ-N	5′ ATAATTTCGCCTATCTCG 3′	a
Af-phnJ-R1	5′ GACCTGCGCCAAATAGCT 3′	a,b
Af-phnJ-D1	5′ ATACCTGGCAGCCCTGCG 3′	d
Af-phnK-N	5′ GAGTTACAGTTCCGTTCC 3′	a
Af-phnK-R1	5′ AATCCAGCAAACCCGCCT 3′	a,b
Af-phnK-D1	5′ AGACGGGAGTAACCGATC 3′	d
Af-phnL-N	5′ ATGAACCTGCTCGAAGTG 3′	a
Af-phnL-R1	5′ GTTTCGCCTCCTGGATCA 3′	a,b
Af-phnL-D1	5′ TCGCGCTATCCTCAGCAT 3′	d
Af-phnM-N	5′ ATGACGCAGGGTTTCAGC 3′	a
Af-phnM-R1	5′ TTTTCTCGCTGTGGGCAT 3′	a,b,c
Af-phnM-D1	5′ TCTGGAAGGCTGGTCAGC 3′	d
Af-phnN-N	5′ CTAACACGCTCATACTGG 3′	a
Af-phnN-R1	5′ TGCTTTCGTGGTAAAGCG 3′	a,b
Af-phnN-D1	5′ CTCAGTTTCGGACGACCA 3′	a
Af-Hip-N	5′ ATGGGCATCCGATCGTCC 3′	a
Af-Hip-R1	5′ TGCCGCACTCTCTGCAAA 3′	a,b
Af-pho84L-Fw1	5′ ACTACGAATACCGGGC 3′	F
Af-pho84L-Rv1	5′ TCGGGTACACGTTCTT 3′	e,f
AfphnGFw1	5′ GGTCTGGGTGATCCCT 3′	f
AfphnGRv1	5′ CTGCTTTATCTGCGTTTCG 3′	e,f
AfphnJFw1	5′ ACTATCGCCCTACACG 3′	f
AfphnJRv1	5′ GTCGGTGAAGTCGAGC 3′	e,f
16S-for	5′ TGGTGCCTAAGCGTACTGAGTGT 3′	f
16S-rev	5′ CCGAAGGGCACTTCCGCA 3′	e,f

[a] a, PCR for DNA macroarray construction; b, Labeled cDNA synthesis for DNA macroarray analysis and PCR for cotranscription experiments; c, cDNA synthesis for cotranscription experiments; d, PCR for cotranscription experiments; e, cDNA synthesis for real-time RT-PCR experiments and PCR amplification for real-time RT-PCR; f, PCR amplification for real-time PCR experiments.

Real time RT-PCR. Primers for real-time reverse transcription-PCR (RT-PCR) were designed with the LightCycler probe design software (Roche). For the cDNA synthesis, 0.5 μg of total RNA was reverse transcribed for 1 h at 42°C by using Moloney murine leukemia virus-RT (Promega) and 10 pmol of an ORF-specific reverse primer. PCR was carried out by using the LightCycler system (Roche). Twenty-microliter reaction mixtures were set up in LightCycler capillaries by using the FastStart DNA Master SYBR green mix. Following the manufacturer's instructions, the reaction mixture contained 1 μl of a 1/20 dilution of the cDNA as the template and 0.5 μl of each corresponding primer. Standard curves for the *pho84l*, *phnG*, and *phnM* genes were constructed by using serial dilutions of genomic DNA from *A. ferrooxidans* ATCC 23270, ranging from 10 ng to 1 pg, as the template and each corresponding primer pair. Thermal cycling conditions were an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 5 s, 60 to 61°C for 5 s, and 72°C for 16 s. Fluorescence measurements were recorded at the end of each extension step.

Search for putative *pho* boxes in *A. ferrooxidans* genome. Sequences 250 bp upstream of the putative *A. ferrooxidans pstS1* and *pstS2* genes were analyzed by using the MAST algorithm (4) for searching conserved motifs. After the identi-

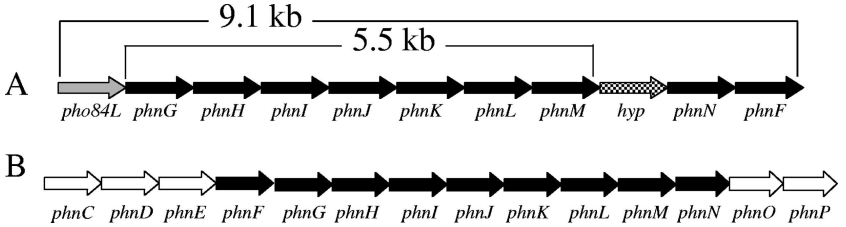

FIG. 1. Comparison of the physical organization of *phn* operons from *A. ferrooxidans* type strain (A) and *E. coli* (B). Black arrows indicate *A. ferrooxidans* genes homologous to *E. coli phn* genes. The white arrows indicate genes from *E. coli phn* genes that are not present in *A. ferrooxidans*. Shaded arrows indicate those *A. ferrooxidans* genes not present in *E. coli*.

fication of two putative motifs related to *pho* boxes in each promoter region of these genes, respectively, these four sequences were aligned using ClustalW. A hidden Markov model was constructed using the HMMER package of programs, version 2.3 (7). Subsequently, the hidden Markov model of *pho* boxes was used to search multiple nonoverlapping matching hits in the complete *A. ferrooxidans* genome sequence (www.tigr.org/db.shtml). After manual inspection of the putative *A. ferrooxidans pho* boxes with respect to their position and distance to their closest gene, 22 were selected for additional analysis.

RESULTS AND DISCUSSION

The *phn* operon from *A. ferrooxidans*. The *phn* orthologs present in *A. ferrooxidans* were located in a putative 9.1-kb operon (Fig. 1). Most *A. ferrooxidans phn* genes showed identities ranging between 33 and 68% to their homologous *phn* genes from other microorganisms. In the *A. ferrooxidans* complete genome sequence database (www.tigr.org), these were annotated as AFE-811 to AFE-821. *E. coli* has a *phn* operon containing 14 genes. Not all of these genes were present in the *phn* operon from *A. ferrooxidans*. Genes *phnGHIJKLM*, encoding the C-P lyase in *E. coli* and other bacteria, were all present in *A. ferrooxidans*. They were located in the same physical order as in *E. coli* (Fig. 1), suggesting the possible existence of a functional enzyme in this acidophilic microorganism. Upstream of the C-P lyase genes from *A. ferrooxidans*, we found an ORF coding for a putative protein showing identity to the P_i transporter Pho84 from *Saccharomyces cerevisiae*, belonging to the major facilitator superfamily. This *A. ferrooxidans* gene was previously referred to as *pho84l* (2). On the other hand, the *phnF* gene from *A. ferrooxidans* was located downstream of the C-P lyase gene cluster. In *E. coli*, PhnF may have a regulatory function since it has amino acid sequences in common with transcription factors that belong to the helix-turn-helix GntR family of bacterial regulators (22). Therefore, a function as a transcriptional repressor might also be speculated for the putative PhnF from *A. ferrooxidans*. The *phnN* gene was also present in the *A. ferrooxidans* genome sequence. In *E. coli*, PhnN is not required for Pn degradation but it was shown to catalyze the formation of ribose-phosphate esters (10), suggesting that its function may be to produce an intermediate during Pn catabolism by the C-P lyase. *A. ferrooxidans* did not possess the genes *phnCDE*, which in *E. coli* code for a putative phosphonate transport system for the utilization of Pn as phosphorus sources (35). The genes *phnO* and *phnP* were also absent in the *A. ferrooxidans* genome. In *E. coli*, PhnO protein may have a regulatory function and PhnP has been described as a C-P lyase accessory protein which is not essential for its enzymatic activity (12).

Figure 1 also shows that the *phn* genes from *A. ferrooxidans* are separated by one gene (*hyp*) encoding a hypothetical protein. The same structure arrangement has been observed in other *phn* gene clusters in several bacteria (11). According to the results of a BLASTP analysis, this ORF codes for a putative protein with 32% identity to DUF 1045 or similar conserved proteins of unknown function present in several members of the family *Rhizobiaceae*. This putative protein must have a conserved function that is most likely nonessential for phosphonate utilization, since its gene is not present in all the genomes coding for C-P lyase (11).

***A. ferrooxidans* can grow using M-Pn or E-Pn as the sole phosphorus sources.** The ability of *A. ferrooxidans* to grow in M-Pn or E-Pn was analyzed (Fig. 2). It is clear that the microorganism was able to grow in both types of Pn as the sole phosphorus sources (Fig. 2A and B). On the other hand, minimal growth of the bacteria was observed in control subcultures carried out in the absence of P_i. Bacterial growth was sustained for more than three subcultures with each of these Pn (data not shown). It has previously been stated that an important physiological feature of phosphonate degradation is the necessary "adaptation" of cells to these compounds. Thus, it has been shown that the degradation of and cell growth on Pn as the sole phosphorus sources begin after a prolonged latent phase, which varies strongly depending on the culture and nature of the phosphonate (12). A similar situation was observed when M-Pn and E-Pn were used by *A. ferrooxidans*, with different lag periods and cell numbers reached during the second subcultures (Fig. 2). The cell numbers attained after growth in the tested Pn were similar to those reached by the standard growth of *A. ferrooxidans* in the presence of P_i (2×10^8 to 4×10^8 cells/ml). Bacteria grew faster during the first subcultures in Pn compared with the second and additional subcultures (data not shown). This may be explained because *A. ferrooxidans* is a polyP-accumulating organism (2), and these polymers are utilized as a phosphorus source during growth in the first subculture in the presence of Pn. In subsequent subcultures, the microorganisms should not have an excess of P_i to store in the form of polyP, as suggested by Fig. 2. To additionally test this idea, we measured polyP levels in cells grown in M-Pn during two subcultures and compared them with those in cells grown in the presence of P_i. Cells grown in the presence of P_i had polyP levels of 55 nmol ATP/mg of protein, whereas M-Pn-grown cells had only 3% of the polyP levels found in control cells. A similar polyP reduction was observed in cells grown in E-Pn (results not shown). The capability to use Pn, which in some environments constitutes a substantial fraction of dissolved organic phosphorus (6, 12, 25), would provide *A. fer-*

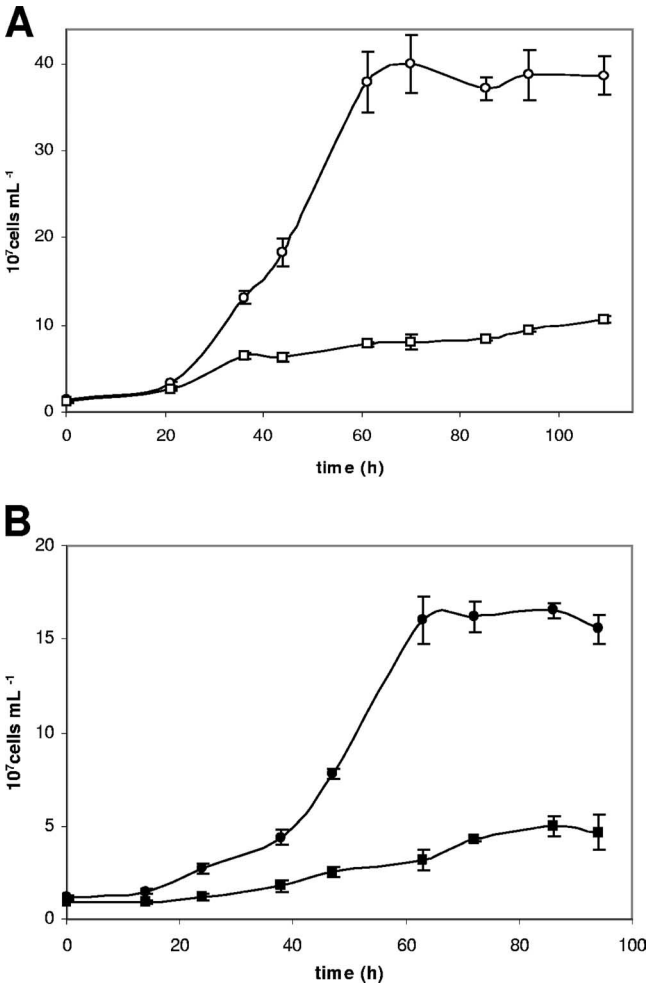

FIG. 2. Growth of *A. ferrooxidans* type strain using Pn as the sole phosphorus source. (A) Cells grown in thiosulfate as the energy source were inoculated in medium in which P_i was replaced by 1 mM M-Pn or in medium with no P_i added as control. Cells grown under these conditions in a first subculture were then inoculated in a second subculture under the same M-Pn concentration as that used in the first subculture (open circles) or in the absence of P_i (open squares). (B) Conditions were the same as described in the legend for panel A, except that E-Pn was used as phosphonate and the cells were grown in ferrous iron as the energy source. After being grown in the last condition for a first subculture, second subcultures were carried out in E-Pn (closed circles) or in the absence of P_i (closed squares). The error bars indicate the standard deviations based on three different experimental values.

rooxidans a competitive advantage in an environment, such as the one found in commercial biomining operations that may periodically experience a scarcity of P_i. Under the presence of excess phosphate, *A. ferrooxidans* accumulates massive amounts of polyP (2), a phenomenon clearly not taking place during growth in the presence of Pn.

The genes of *A. ferrooxidans* coding for C-P lyase are cotranscribed. A cotranscription experiment was carried out (Fig. 3) in which the cDNA was obtained by using RNA extracted from an M-Pn-grown culture by using a reverse primer hybridizing with the *phnM* gene. PCR amplifications were carried out by using this cDNA as the template and each pair of primers lying in adjacent genes. The presence of an amplicon indicated that this pair of genes was part of a polycistronic messenger. Positive results were obtained for all the pairs of genes from *phnG* to *phnM*, with the expected product sizes for each PCR. These results clearly show that the *A. ferrooxidans* genes coding for C-P lyase were expressed in the form of a transcriptional unit composed of at least seven genes, from *phnG* to *phnM*. Gene *pho84l* gave a very faint amplification product under the same conditions (data not shown), suggesting that it might also be part of this transcriptional unit (see below).

Transcription of *A. ferrooxidans phn* genes is increased when M-Pn is used as the sole P_i source during growth. To determine the expression of the C-P lyase genes in cultures of *A. ferrooxidans* grown in M-Pn, a DNA macroarray experiment was performed as described previously (1, 8, 30). Quantification of the spots obtained showed a strong induction of the transcript levels for all the *phn* genes in the membrane hybridized with cDNAs synthesized from the culture grown in M-Pn compared with the levels obtained from a control culture grown in the presence of P_i (Fig. 4). Interestingly, *hyp* gene expression was the most strongly up-regulated in the presence of M-Pn. However, at present we have no additional data that may indicate a role for this conserved hypothetical protein in phosphonate metabolism in *A. ferrooxidans*. *pho84l* was also induced under these conditions, in agreement with the idea that this gene encodes a putative P_i and/or Pn permease which may be part of the same transcriptional unit as that for C-P lyase. The level of expression of *pstS2*, which is present in a genomic context different from that of *phn* genes, also increased in cells grown in M-Pn, indicating that when cells of *A. ferrooxidans* grew in 1 mM M-Pn, they were still under P_i starvation conditions. Under these circumstances, the Pho regulon would be activated and, hence, the synthesis of C-P lyase would be induced in agreement with the observed results (Fig. 4).

To validate some of these results, we used real-time RT-PCR. Standard calibration curves were constructed with genomic DNAs for *pho84l*, *phnG*, and *phnJ*. The analysis of the corresponding melting curves revealed the presence of unique amplicons for each gene with melting temperature values of 91.2°C for *pho84l*, 88°C for *phnG*, and 87.1°C for *phnJ*. When the bacterium was grown in the presence of 1 mM M-Pn, an increase in transcript level was found by real-time RT-PCR for *pho84l*, *phnG*, and *phnJ* (Table 2). The results obtained by DNA macroarrays and real-time RT-PCR confirmed that *phn* gene expression was induced in cultures grown in M-Pn as the sole phosphorus source.

Phosphonate metabolism and its possible relationship with the Pho regulon of *A. ferrooxidans*. In *E. coli* and other bacteria, Pn metabolism is induced as part of the Pi starvation response known as the Pho regulon (33, 34). We have previously shown that *A. ferrooxidans* possesses genes homologous to those of *E. coli* Pho regulon components (31). *A. ferrooxidans* has two *pstS* genes, which we have named *pstS1* and *pstS2* based on the percent identities of the putative PstS1 and PstS2 proteins with their *E. coli* homologues. PstS is the periplasmic P_i binding protein (16) and is one of the most-induced proteins during the P_i starvation response in *E. coli* and many other bacteria. Two *pho* boxes were found in each promoter se-

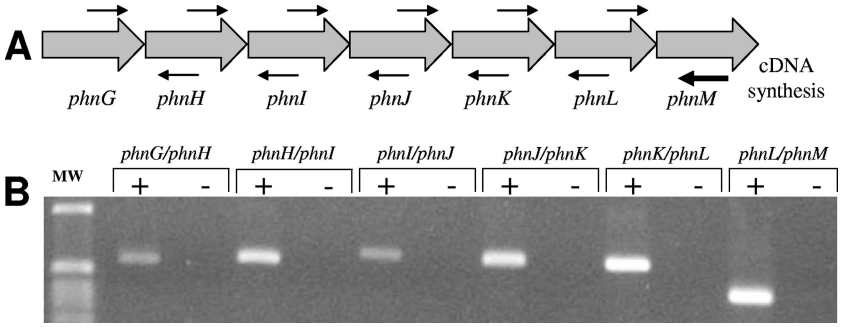

FIG. 3. *A. ferrooxidans phn* genes are cotranscribed. (A) cDNA was synthesized with a reverse primer hybridizing to the *phnM* gene (thicker black arrow) and *A. ferrooxidans* total RNA extracted at the exponential phase from a culture grown in M-Pn. (B) PCR amplifications were carried out with this cDNA and each corresponding primer pair (thin black arrows). Determination of the cotranscription for each pair of adjacent genes was carried out by using primers labeled D1 (upstream genes) and R1 (downstream genes) in Table 1. The results shown were obtained after 32 PCR cycles. PCRs using cDNA templates synthesized in the presence (+) or absence (−) of reverse transcriptase to detect the possible presence of genomic DNA contamination are shown.

quence of *pstS1* and *pstS2* (results not shown). By using these sequences, we constructed a hidden Markov model and after searching the entire genome sequence of *A. ferrooxidans* for the presence of *pho* boxes, we found a putative *pho* box in the upstream regions of the C-P lyase operon (data not shown). This evidence strongly suggests that as in *E. coli* and other bacteria, C-P lyase expression forms part of the Pho regulon in *A. ferrooxidans*.

In our studies of the phosphate starvation response of *A. ferrooxidans*, we have determined that the biosynthesis of the PstS2 protein was highly induced in response to P_i starvation (31). A 10-fold induction of this transcript was also observed in cultures grown in M-Pn as the only phosphorus source (Fig. 4), most likely due to a Pi starvation condition, as mentioned previously.

Surprisingly, we did not find a Pit-like transporter in this bacterium; instead we found an ORF coding for a protein similar to the Pho84 P_i transporter from *S. cerevisiae* (2). Pho84 and Pho89 are the major P_i transporters in the yeast *S. cerevisiae*. Pho84, like Pit, belongs to the family of P_i:H^+ symporters and is a member of the major facilitator superfamily. The Pho84 transporter is functional only in acidic environmental conditions (19). It is remarkable that *A. ferrooxidans*, being an acidophilic microorganism, possesses a putative yeast Pho84 P_i transporter. In this regard, only proteins similar to Pho84, but not to Pit (or Pho89) are present in the genomes of other acidophilic microorganisms (2). Since *A. ferrooxidans* does not possess the genes *phnCDE*, which in *E. coli* code for a putative phosphonate transport system for the utilization of Pn as phosphorus sources, it is tempting to speculate that *pho84*, whose expression was also induced in the presence of phosphonate (Table 2), may also be able to transport these compounds to the interior of *A. ferrooxidans*. In this connection, it is known that a strain of the yeast *Kluyveromyces fragilis* is able to utilize an organophosphonate as a nitrogen source (26).

Under P_i limitation conditions, most *A. ferrooxidans phn* genes showed a rather limited degree of induction under our experimental conditions (data not shown). This result, compared with the strong induction of these genes in the presence of M-Pn (Fig. 4), suggests that in addition to P_i starvation, Pn themselves may also play a role in the induction of the genes that the cell expresses to cope with their presence. In this regard, it has recently been shown that *N*-(phosphonometyl) glycine (glyphosate) induces the expression of the gene coding for a porin-like protein in *Bradyrhizobium* sp. (*Lupinus*) (5). Obviously, additional genetic experiments would be required to verify our suggestions. Unfortunately, this is not currently feasible, as at the time of writing, there are no efficient genetic systems for *A. ferrooxidans* to obtain mutants in each of these genes to demonstrate the functional genetic controls during P_i starvation and Pn metabolism responses.

Presence of *phn* genes in other biomining organisms: genomic and environmental aspects. Pn are ubiquitous in nature, but not all microorganisms possess the genetic capabilities needed to degrade and use them as phosphorus sources. A search for *phn* genes in 140 complete bacterial genomes

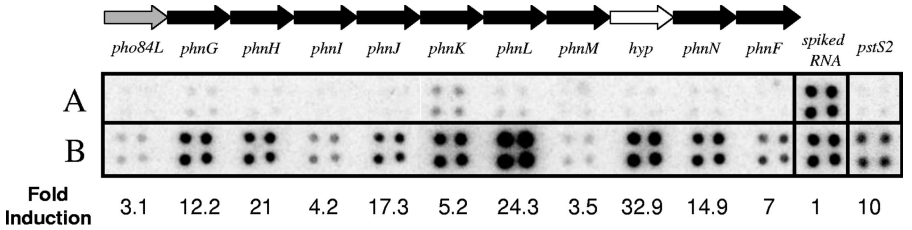

FIG. 4. Induction of expression of *phn* genes in *A. ferrooxidans* type strain as determined by DNA macroarray experiments. (A) Control membrane hybridized with cDNA prepared from a culture grown in the presence of P_i. (B) Membrane hybridized with cDNA obtained from a culture grown in 1 mM M-Pn. Numbers under the spots indicate the increase in the transcript levels for each gene expressed as the ratio of normalized intensities.

TABLE 2. Induction of transcript levels for some *phn* genes from the type strain of *A. ferrooxidans* as determined by real-time RT-PCR[a]

Gene	Value obtained with M-Pn		Value obtained with P_i		Induction ratio
	Ct	Relative copy number	Ct	Relative copy number	
pho84L	28.1	4.6×10^5	31	9×10^4	7
phnG	21	9.6×10^6	24.9	7.8×10^5	8
phnJ	20.9	8.1×10^6	26.1	2.5×10^5	23
16S rRNA	10.2	6.9×10^7	9.5	4.7×10^7	1

[a] The induction ratios were determined by comparing the relative transcript cDNA levels from *A. ferrooxidans* grown in M-Pn versus those obtained in control cells grown in the presence of P_i.

showed that homologous clusters for C-P lyase have great structural and compositional variation among microorganisms, suggesting that these degradative pathways have been subjected to extensive lateral gene transfers during their evolution (11). Nevertheless, *phnGHIJKLM* genes, which are essential for Pn cleavage, are generally linked together. Recently, it was reported that the presence of C-P lyase genes would confer an ecological advantage to *Trichodesmium* sp. in their colonization of marine environments, where P_i is generally present in nanomolar concentrations. It was demonstrated that these genes are expressed under P_i starvation conditions, and similar results were obtained with environmental *Trichodesmium* populations isolated from the western North Atlantic (6).

To our knowledge, this is the first report of an acidophilic chemolithoautotrophic microorganism that is able to use Pn as a valuable phosphorus source. We undertook a search for C-P lyase genes in the total and partial genomes of other biomining microorganisms from environmental samples covering mine drainage metagenome, an acid mine drainage biofilm community (15, 29) and the complete genome of *Ferroplasma acidarmanus* (9). However, we did not find genes related to phosphonate degradation in these microorganisms. When more genome sequences from biomining microorganisms are available, it will be of great interest to search for the presence of C-P lyase genes. This search will allow us to determine whether their presence is a unique characteristic of *A. ferrooxidans* or whether they are also present in other acidophilic microorganisms, conferring all of them the advantage of accessing the unique phosphorus pool derived from Pn in their extreme environments.

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REFERENCES

1. **Acosta, M., S. Beard, J. Ponce, M. Vera, J. C. Mobarec, and C. A. Jerez.** 2005. Identification of putative sulfurtransferase genes in the extremophilic *Acidithiobacillus ferrooxidans* ATCC 23270 genome: structural and functional characterization of the proteins. OMICS **9:**13–29.
2. **Alvarez, S., and C. A. Jerez.** 2004. Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*. Appl. Environ. Microbiol. **70:**5177–5182.
3. **Ault-Riché, D., C. D. Fraley, C. M. Tzeng, and A. Kornberg.** 1998. Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. J. Bacteriol. **180:**1841–1847.
4. **Bailey, T. L., and M. Gribskov.** 1998. Combining evidence using p-values: application to sequence homology searches. Bioinformatics **14:**48–54.
5. **de María, N., A. Guevara, M. T. Serra, I. García-Luque, A. González-Sama, M. García de Lacoba, M. R. de Felipe, and M. Fernández-Pascual.** 2007. Putative porin of *Bradyrhizobium* sp. (*Lupinus*) bacteroids induced by glyphosate. Appl. Environ. Microbiol. **73:**5075–5082.
6. **Dyhrman, S. T., P. D. Chappell, S. T. Haley, J. W. Moffett, E. D. Orchard, J. B. Waterbury, and E. A. Webb.** 2006. Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature **439:**68–71.
7. **Eddy, S. R.** 1998. Profile hidden Markov models. Bioinformatics **14:**755–763.
8. **Farah, C., M. Vera, D. Morin, D. Haras, C. A. Jerez, and N. Guiliani.** 2005. Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. Appl. Environ. Microbiol. **71:**7033–7040.
9. **Gihring, T. M., P. L. Bond, S. C. Peters, and J. F. Banfield.** 2003. Arsenic resistance in the archaeon *Ferroplasma acidarmanus*: new insights into the structure and evolution of the *ars* genes. Extremophiles **7:**123–130.
10. **Hove-Jensen, B., T. J. Rosenkrantz, A. Haldimann, and B. L. Wanner.** 2003. *Escherichia coli phnN*, encoding ribose 1,5-bisphosphokinase activity (phosphoribosyl diphosphate forming): dual role in phosphonate degradation and NAD biosynthesis pathways. J. Bacteriol. **185:**2793–2801.
11. **Huang, J., Z. Su, and Y. Xu.** 2005. The evolution of microbial phosphonate degradative pathways. J. Mol. Evol. **61:**682–690.
12. **Kononova, S. V., and M. A. Nesmeyanova.** 2002. Phosphonates and their degradation by microorganisms. Biochemistry **67:**184–195.
13. **Kornberg, A., N. N. Rao, and D. Ault-Riché.** 1999. Inorganic polyphosphate: a molecule of many functions. Annu. Rev. Biochem. **68:**89–125.
14. **La Nauze, J. M., H. Rosenberg, and D. C. Shaw.** 1970. The enzymatic cleavage of the carbon-phosphorus bond: purification and properties of phosphonatase. Biochim. Biophys. Acta **212:**332–350.
15. **Lo, I., V. J. Denef, N. C. Verberkmoes, M. B. Shah, D. Goltsman, G. DiBartolo, G. W. Tyson, E. E. Allen, R. J. Ram, J. C. Detter, P. Richardson, M. P. Thelen, R. L. Hettich, and J. F. Banfield.** 2007. Strain-ressolved community proteomics reveals recombining genomes of acidophilic bacteria. Nature **446:**537–541.
16. **Luecke, H., and F. A. Quiocho.** 1990. High specificity of a phosphate transport protein determined by hydrogen bonds. Nature **347:**402–406.
17. **McMullan, G., and J. P. Quinn.** 1994. In vitro characterization of a phosphate starvation-independent carbon-phosphorus bond cleavage activity in *Pseudomonas fluorescens* 23F. J. Bacteriol. **176:**320–324.
18. **McMullan, G., R. Watkins, D. B. Harper, and J. P. Quinn.** 1991. Carbon-phosphorus bond cleavage activity in cell-free extracts of *Enterobacter aerogenes* ATCC 15038 and *Pseudomonas* sp. 4ASW. Biochem. Int. **25:**271–279.
19. **Persson, B. L., J. Petersson, U. Fristedt, R. Weinander, A. Berhe, and J. Pattison.** 1999. Phosphate permeases of *Saccharomyces cerevisiae*: structure, function and regulation. Biochim. Biophys. Acta **1422:**255–272.
20. **Ramirez, P., H. Toledo, N. Guiliani, and C. A. Jerez.** 2002. An exported rhodanese-like protein is induced during growth of *Acidithiobacillus ferrooxidans* in metal sulfides and different sulfur compounds. Appl. Environ. Microbiol. **68:**1837–1845.
21. **Rawlings, D. E.** 2002. Heavy metal mining using microbes. Annu. Rev. Microbiol. **56:**65–91.
22. **Rigali, S., M. Schlicht, P. Hoskisson, H. Nothaft, M. Merzbacher, B. Joris, and F. Titgemeyer.** 2004. Extending the classification of bacterial transcription factors beyond the helix-turn-helix motif as an alternative approach to discover new *cis*/*trans* relationships. Nucleic Acids Res. **32:**3418–3426.
23. **Rohwerder, T., T. Gehrke, K. Kinzler, and W. Sand.** 2003. Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl. Microbiol. Biotechnol. **63:**239–248.
24. **Seeger, M., and C. A. Jerez.** 1993. Phosphate-starvation induced changes in *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. **108:**35–41.
25. **Ternan, N., J. McGrath, G. McMullan, and J. Quinn.** 1998. Review: organophosphonates: occurence, synthesis and biodegradation by microorganisms. World J. Microbiol. Biotechnol. **14:**635–647.
26. **Ternan, N., and G. McMullan.** 2000. The utilization of 4-aminobutylphosphonate as sole nitrogen source by a strain of *Kluyveromyces fragilis*. FEMS Microbiol. Lett. **184:**237–240.
27. **Ternan, N. G., and J. P. Quinn.** 1998. *In vitr*o cleavage of the carbon-phosphorus bond of phosphonopyruvate by cell extracts of an environmental *Burkholderia cepacia* isolate. Biochem. Biophys. Res. Commun. **248:**378–381.
28. **Torriani-Gorini, A. Y., E. Yagil, and S. Silver (ed.).** 1994. Phosphate in microorganisms: cellular and molecular biology. American Society for Microbiology, Washington, DC.
29. **Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield.**

2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature **428:**37–43.
30. **Valenzuela, L., A. Chi, S. Beard, A. Orell, N. Guiliani, J. Shabanowitz, D. F. Hunt, and C. A. Jerez.** 2006. Genomics, metagenomics and proteomics in biomining microorganisms. Biotechnol. Adv. **24:**197–211.
31. **Vera, M., N. Guiliani, and C. A. Jerez.** 2003. Proteomic and genomic analysis of the phosphate starvation response of *Acidithiobacillus ferrooxidans*. Hydrometallurgy **71:**125–132.
32. **Wackett, L. P., S. L. Shames, C. P. Venditti, and C. T. Walsh.** 1987. Bacterial carbon-phosphorus lyase: products, rates, and regulation of phosphonic and phosphinic acid metabolism. J. Bacteriol. **169:**710–717.
33. **Wanner, B. L.** 1994. Molecular genetics of carbon-phosphorus bond cleavage in bacteria. Biodegradation **5:**175–184.
34. **Wanner, B. L.** 1996. Signal transduction in the control of phosphate-regulated genes of *Escherichia coli*. Kidney Int. **49:**964–967.
35. **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.