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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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 hydrolyase, which cleaves specifically phosphonoacetate (17); phosphonoxyruvate hydrolyase (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, phnGHIJKLMNOP, 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 (Pₐ) may therefore greatly affect the biodegradation of minerals (21). Bacteria meet their phosphorus requirements through the assimilation of Pₐ 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).
We have previously studied the P 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, 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, these expression levels of C-P lyase genes when the microorganism could be utilized 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) as described before (20) or in ferrous sulfate containing 9K medium (0.17 mM P). In spite of the different P concentrations, both media had an excess of P, for the growth of the microorganisms and they were therefore considered a “presence of P” condition. In control cultures, the P salt was not included in the corresponding media. For growth in M-Pn or E-Pn, the P 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 Lumix/96). Concentration of polyP is given in terms of P concentrations.

**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, by using the ImPromII RT system (Promega), a mixture of specific oligonucleotides for the open reading frames (ORFs) of interest and a [α-32P]dCTP (Amersham Bio-sciences). 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).

### 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 45 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* *phoS1* and *phoS2* genes were analyzed by using the MAST algorithm (4) for searching conserved motifs. After the identi-
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 P4 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 Pn. 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 P1 (2 × 10^6 to 4 × 10^6 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 phosphorous source during growth in the first subculture in the presence of Pn. In subsequent subcultures, the microorganisms should not have an excess of P1 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 P1. Cells grown in the presence of P1 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-
a competitive advantage in an environment, such as the one found in commercial biomining operations that may periodically experience a scarcity of P. 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 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 (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 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* grow in 1 mM M-Pn, they were still under P 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 increased 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 binding protein (16) and is one of the most-induced proteins during the P starvation response in *E. coli* and many other bacteria. Two *pho* boxes were found in each promoter se-
sequence 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 Pi 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 Pi transporter from *S. cerevisiae* (2). Pho84 and Pho89 are the major Pi 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-(phosphonomethyl) 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...
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, phnGHJKLM 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 is generally present in nanomolar concentrations. It was demonstrated that these genes are expressed under P 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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