Proteomic and genomic analysis of the phosphate starvation response of *Acidithiobacillus ferrooxidans*

M. Vera, N. Guiliani, C.A. Jerez*

Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, The Millennium Institute for Advanced Studies in Cell Biology and Biotechnology, University of Chile, Santiago, Chile

Abstract

The recent availability of an incomplete genomic sequence from *Acidithiobacillus ferrooxidans* allowed us to continue and strengthen the demanding task of investigating the proteome and its functional implications in this extremophilic microorganism. The proteins of *At. ferrooxidans* were separated by two-dimensional polyacrylamide gel electrophoresis and their levels of synthesis and the microsequencing of their N-terminal end amino acids were determined. To link the 2D gel spots of interest with the genes that encodes them, we studied the global changes in gene expression of *At. ferrooxidans* when the bacterium was confronted with phosphate starvation. By comparing the amino acid sequences of the proteins whose synthesis was induced or repressed under these conditions, with the available *At. ferrooxidans* genomic database, we found several putative genes whose expression may be related to phosphate starvation. Analysis of the genome DNA sequences upstream and downstream of these genes showed us details of the structure of putative operons present in *At. ferrooxidans*, strongly suggesting the existence of a Pho regulon containing the putative genes *phoB*, *phoR*, *pstS*, *pstC*, *pstA*, *pstB*, *phoU*, *ppx* and *ppk*. Some differences were seen in the organization of the genes in the possible Pho regulon of *At. ferrooxidans* when compared with the Pho operons from other microorganisms. This was specially evident in the organization of the genes involved in polyphosphate metabolism (*ppk* and *ppx*). Regulation of phosphate metabolism is of particular relevance when *At. ferrooxidans* grows in the presence of arsenopyrites, which release arsenate, a structural analog of phosphate. Structural comparison between the specific phosphate-binding protein PstS from *Escherichia coli* and the corresponding *At. ferrooxidans* homolog showed that both proteins are highly conserved, including the phosphate/arsenate binding site, which shares seven of the eight amino acid residues necessary for the hydrogen bonding to the four oxygens of phosphate.

Keywords: Proteomic analysis; Phosphate starvation; *Acidithiobacillus ferrooxidans*

1. Introduction

Acidithiobacillus ferrooxidans (formerly *Thiobacillus ferrooxidans*) is an important bacterium for the bioleaching of ores (Rawlings, 1997; Tuovinen, 1990; Lundgren and Silver, 1980). During this process, changes in several conditions, for instance nutrient starvation, may adversely affect the microorganisms. Inorganic phosphate (Pi) is an essential nutrient for all living cells. Therefore, the lack of phosphate may affect the bioleaching of minerals (Tuovinen, 1990; Matin, 1991; Beck and Shafia, 1964). The physiolog-

* Corresponding author.
E-mail address: cjerez@uchile.cl (C.A. Jerez).

ical response to phosphate deprivation has been well characterized in *Escherichia coli* (Wanner, 1996; Torriani-Gorini, 1994), which possesses an emergency system known as the Pho regulon (Wanner, 1996; Torriani-Gorini, 1994; Makino et al., 1994), consisting of a number of genes coding for proteins that allow the bacteria to scavenge traces of usable phosphate sources from the environment. Responses include the production of proteins within the outer membrane and interior membrane that transport phosphate into the cytoplasm.

Some of these Pi starvation-induced proteins are the outer membrane pore protein PhoE, alkaline phosphatase (PhoA), the PhoR/PhoB two-component system controlling the response: PhoR being the sensor and PhoB the response regulator. In *E. coli*, PhoB activates the transcription of the genes belonging to the Pho regulon by recognizing and binding to the upstream regulatory element known as the Pho box. Several other proteins (PstA, PstB, PstC, PstS), which are part of the high affinity phosphate-specific transport (Pst) system, are also induced in their synthesis as part of the response to phosphate starvation (Wanner, 1996; Torriani-Gorini, 1994; Makino et al., 1994). Additionally, bacteria synthesize inorganic polyphosphates (polyP) as a Pi reservoir (Kornberg et al., 1999). The enzyme responsible for the synthesis of polyP is the polyphosphate kinase (PPK), and the enzyme degrading polyP to Pi is the exopolyphosphatase (PPX). Both genes (*ppk* and *ppx*, respectively) are apparently part of the Pho regulon in *E. coli* (Torriani-Gorini, 1994).

We have previously analyzed the global protein changes in *At. ferrooxidans* when subjected to phosphate starvation (Seeger and Jerez, 1993; Jerez et al., 1992). By two-dimensional polyacrylamide gel electrophoresis, at least 25 proteins changed their levels of expression under the limiting conditions (Seeger and Jerez, 1992, 1993). Recently, we have also developed an assay based on the induction of outer membrane proteins during phosphate starvation to determine in situ the phosphate-lacking state of *At. ferrooxidans* (Varela et al., 1998). The determination of this physiological state is particularly important when the microorganisms are grown in the presence of arsenate. Being a structural analog of phosphate, arsenate enters the cell through both the Pit (phosphate inorganic transporter, constitutive system) and the Pst (inducible) systems employed to incorporate phosphate (Van Veen, 1997; Luecke and Quiocho, 1990). As a consequence, the resistance of *At. ferrooxidans* to arsenate will greatly depend not only in having an arsenic resistance *ars* operon (Nies and Silver, 1995) but also on the phosphate concentration present in the growth medium (Varela et al., 1998).

In the present report, we illustrate the use of reverse genetics and the available genomic database to analyze the putative genes of *At. ferrooxidans* whose expression may be related to phosphate starvation. Starting with the amino acid N-terminal sequence of a protein induced under phosphate starvation, we found it to be highly similar to PstS, the phosphate binding protein of the (ABC type) Pst phosphate transporter. The analysis of the genome DNA sequences upstream and downstream of the putative *At. ferrooxidans pstS* gene strongly suggests the existence of a Pho regulon in this bacterium. On the other hand, the database currently available showed that a Pit system was not apparent in this microorganism.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The *At. ferrooxidans* strain ATCC 19859 was used in these studies. Growth on ferrous iron was in modified 9K medium as previously described (Amaro et al., 1998).

2.2. Polyacrylamide gel electrophoresis

Standard two-dimensional polyacrylamide gel electrophoresis (2D PAGE) (pH 5 to 7 in the first dimension) or non-equilibrium pH (2D NEPHGE) (pH 3 to 10 in the first dimension) (O'Farrell et al., 1977) was performed as described before for *At. ferrooxidans* (Seeger and Jerez, 1992, 1993; Jerez et al., 1992).

2.3. N-terminal end amino acid sequencing

Total *At. ferrooxidans* proteins were separated by 2D PAGE and the spots of interest were cut out from the gels and transferred to PVDF membranes for determination of the N-terminal amino acid sequence (Jerez et al., 1992). Some sequencing was performed by the Lab.

de Microséquenç age des Protéines of the Institut Pasteur Laboratory (Paris) and others performed at the sequencing facilities of the GBF, Germany.

2.4. Sequence and database analysis

A tBLASTx (http://www.ncbi.nlm.nih.gov/blast) search against the *At. ferrooxidans* ATCC 23270 unfinished genome sequence database (The Institute for Genomic Research, http://www.tigr.org) was run using the N-terminal sequence of the protein of interest. After the identification of the contig containing the gene of interested we looked for the upstream and downstream sequences in a kind of "internal contig walking". In each iteration we obtained 1 kb of new sequences from this contig. The resulting sequences were merged by using the GCG set of programs. The ORFs, corresponding to homologs of the genes analyzed were searched for by using the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The physicochemical properties of the proteins derived from the ORF finder program were obtained using the PROTOPARAM program at the Expasy molecular biology site (http://expasy.cbr.nrc.ca/tools/protparam.html). The amino acidic sequence alignment between a given *E. coli* gene and its *At. ferrooxidans* homolog was performed with Clustal W (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and edited by using the BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html). Structural alignments between the PstS protein and its *At. ferrooxidans* homolog were done using the available structure and the Cn3D program (http://www.ncbi.nlm.nih.gov/Structure).

3. Results and discussion

3.1. Proteomic analysis of At. ferrooxidans

Total protein from *At. ferrooxidans* from different growth conditions were separated by 2D NEPHGE. Fig. 1A shows the gel protein pattern obtained for *At. ferrooxidans* ATCC 19859. The proteins were stained with Coomassie Blue and these gels were the starting point to isolate proteins of interest that change their levels under different growth conditions (Seeger and Jerez, 1992, 1993; Jerez et al., 1992; Varela et al., 1998). Although the sensitivity and resolution of this type of gels does not allow the separation of all the proteins from *At. ferrooxidans*, the method is sensitive enough to start the proteomic analysis of a number of gene products.

By employing this 2D PAGE procedure we have previously identified several proteins induced or repressed under a variety of conditions as indicated in Fig. 1A (Seeger and Jerez, 1992, 1993; Jerez et al., 1992; Varela et al., 1998). Some of these proteins are the molecular chaperones DnaK (sp/P29133) and GroEL (sp/P29134) induced under heat shock, ethanol and pH changes (Amaro et al., 1998; Varela and Jerez, 1992), the outer membrane protein Omp40 (bbs/120030; emb/CAA10107 and AJ012661) changing under phosphate starvation (Jerez et al., 1992; Guiliani and Jerez, 2000) and rusticyanin (Ru), induced by the presence of ferrous iron and repressed by the presence of sulfur (Osorio et al., 1993). As seen in Fig. 1B,C, the inset window shows the change in the levels of synthesis of several proteins when the cells were subjected to phosphate starvation. Protein 16 synthesis increased due to the lack of phosphate, and was therefore isolated from the 2D gel and subjected to N-terminal amino acid sequencing, resulting in the sequence $_{1}$APTISLLETGSTLLYPLFNLAV$_{22}$.

Comparison of this amino acid sequence with the GeneBank database showed that this protein was similar to PstS. This result was not surprising, since protein 16 was induced in the absence of phosphate, as is PstS from *E. coli* together with the other proteins of the Pho regulon (Torriani-Gorini, 1994). This result strongly suggests that *At. ferrooxidans* has a phosphate scavenging system similar to the one present in *E. coli* and other bacteria. In this regard, we have previously detected changes in the outer membrane proteins of *At. ferrooxidans* when the cells were subjected to phosphate starvation (Jerez et al., 1992; Varela et al., 1998).

3.2. Location of the pstS-like sequence in the genome of At. ferrooxidans

To locate the putative *pstS* gene in *At. ferrooxidans*, we run a tBLASTx search against the *At.*

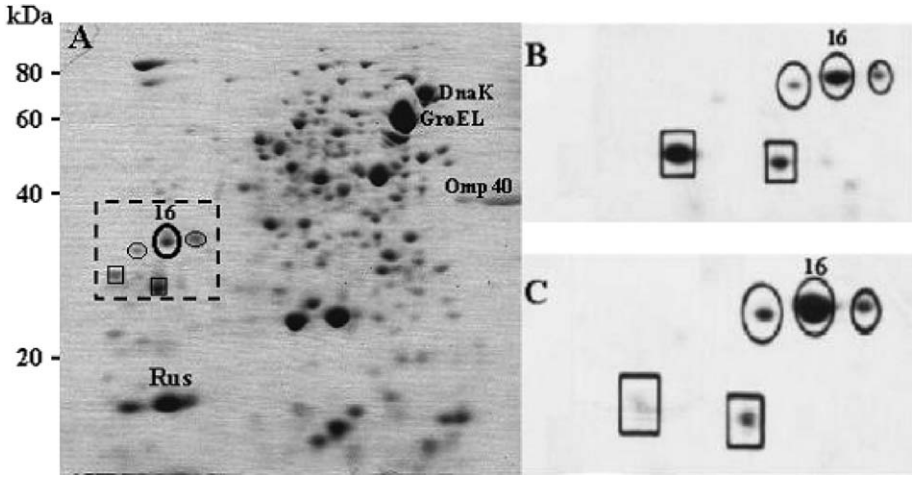

Fig. 1. 2D NEPHGE of total proteins from *At. ferrooxidans*. (A) Total protein from *At. ferrooxidans* ATCC 19859 separated by 2D PAGE and stained with Coomassie Blue. The inset indicates a group of proteins of which spot 16 was further analyzed in cells grown in the presence of phosphate (B) and in phosphate-starved cells (C). Numbers to the left indicate molecular weight markers in kilodaltons.

ferrooxidans ATCC 23270 unfinished genome sequence database using the N-terminal amino acid sequence of the PstS protein obtained or the sequence of PstS from *E. coli*. We found two different contigs in the genome from *At. ferrooxidans*, containing significant scores with the *E. coli* PstS sequence, suggesting the possible existence of two *pstS*-like genes in *At. ferrooxidans* (*pstS1* and *pstS2*). The *pstS2* gene contained the N-terminal sequence obtained from protein 16. With the current available information, we considered the *pstS1* gene with highest score the possible *pstS* homolog. Fig. 2 shows the entire amino acid sequence of the putative *At. ferrooxidans* PstS1 compared with that of *E. coli* PstS.

Both *At. ferrooxidans pstS* genes showed a putative signal peptide, supporting the putative periplasmic location of the proteins, as found for the *E. coli* PstS. The putative PstS1 amino acid sequence showed an identity of 50% and a similarity of 64% with the *E. coli* PstS protein.

All Pho regulon genes or operons are preceded by a promoter containing an upstream activation site with a consensus Pho box sequence for transcriptional activation by phosphorylated PhoB (Makino et al., 1994). Also, all Pho regulon promoters have a number of features in common. The Pho box is composed of two 7-bp direct repeats with the well conserved consensus CTGTCAT separated by a 4-bp segment that is part of the −35 region (Makino et al., 1986). As Fig. 3 shows, the putative gene coding for PstS1 from *At. ferrooxidans* shows a possible 18-bp Pho box with the aforementioned characteristics. In the case of the putative gene for PhoB from *At. ferrooxidans*, although less conserved, a possible Pho box is also apparent.

3.3. Structural comparison of *At. ferrooxidans* PstS-like protein with *E. coli* PstS

The high degree of conservation between the *E. coli* and the putative *At. ferrooxidans* PstS, and the fact that a crystallographic structure for the *E. coli* PstS protein has been reported (Luecke and Quiocho, 1990), prompted us to do a structural alignment between the two proteins. Fig. 4A,B shows the results of this comparison. There is a high degree of structural conservation between the two proteins, indicating that both may have the same function in binding phosphate and its structural analog arsenate.

The degree of structural similarity between the PstS from *E. coli* and that of the putative *At. ferrooxidans* PstS is more evident when the active site for the phosphate binding is compared. Seven of the eight residues necessary for the hydrogen bond-

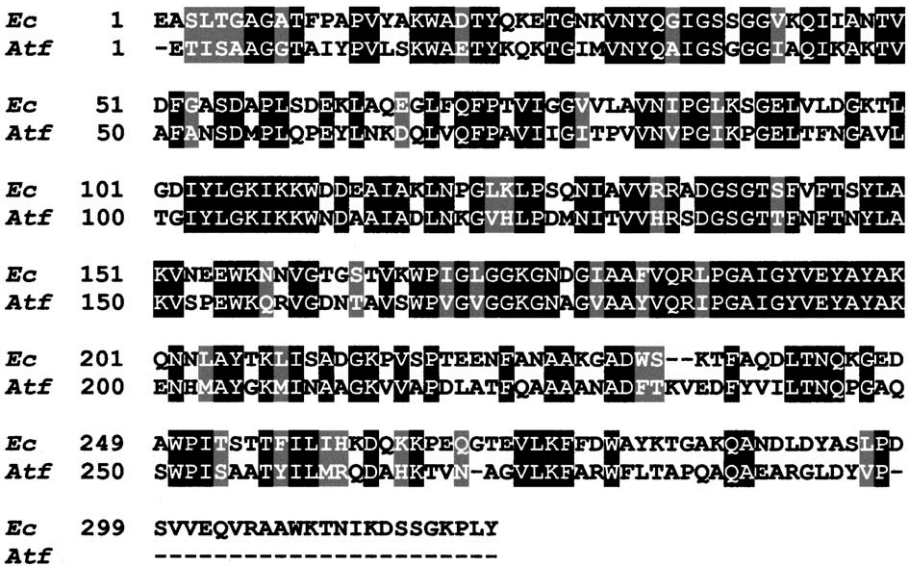

Fig. 2. Amino acid sequence alignment between *E. coli* (*Ec*) PstS protein and its *At. ferrooxidans* (*Atf*) homolog PstS1. This alignment was done excluding the signal peptide necessary for the periplasmic export of these proteins. Black boxes, identity; gray boxes, similarity.

ing to the four oxygens of phosphate are conserved. These residues include Thr 10, Arg 135, Ser 139, Thr141, Ser 38, Gly 140 and Asp56, as shown in Fig. 4C.

3.4. In silico search of a putative Pho regulon containing the pstS-like gene in the At. ferrooxidans genome

After locating part of the contig containing the *pstS*-like gene with a high degree of homology to the *E. coli pstS*, we searched for the upstream and downstream sequences (see Materials and methods). A tBLASTx search was run against the incomplete genome sequence of the *At. ferrooxidans* ATCC 23270. After running the program ORF Finder with an 8.2 kb of the sequence, we identified eight ORFs that are summarized in Table 1. In general, the molecular sizes of all the proteins from *At. ferrooxidans* that had putative homologs in the Pho regulon from *E. coli* were very similar. The theoretical isoelectric points showed a greater variation, specially in the case of PhoR. Some of these differences may reflect adaptations of the proteins which are or have a region in the acidic periplasmic space of *At. ferrooxidans*.

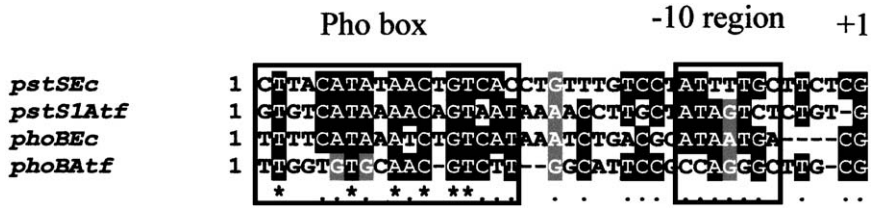

Fig. 3. Possible existence of Pho boxes in the promoter regions of *phoB* and *pstS* from *At. ferrooxidans*. The nucleotide sequence alignments between the *pstS1* (*pstS1Atf*) and *phoB* putative promoter regions of *At. ferrooxidans* (*phoBAtf*) are compared with the corresponding Pho boxes from the homolog genes from *E. coli* (*pstSEc* and *phoBEc*). The known −10 regions and the mRNA start sites (a G at +1) described for *E. coli* are indicated (Makino et al., 1994).

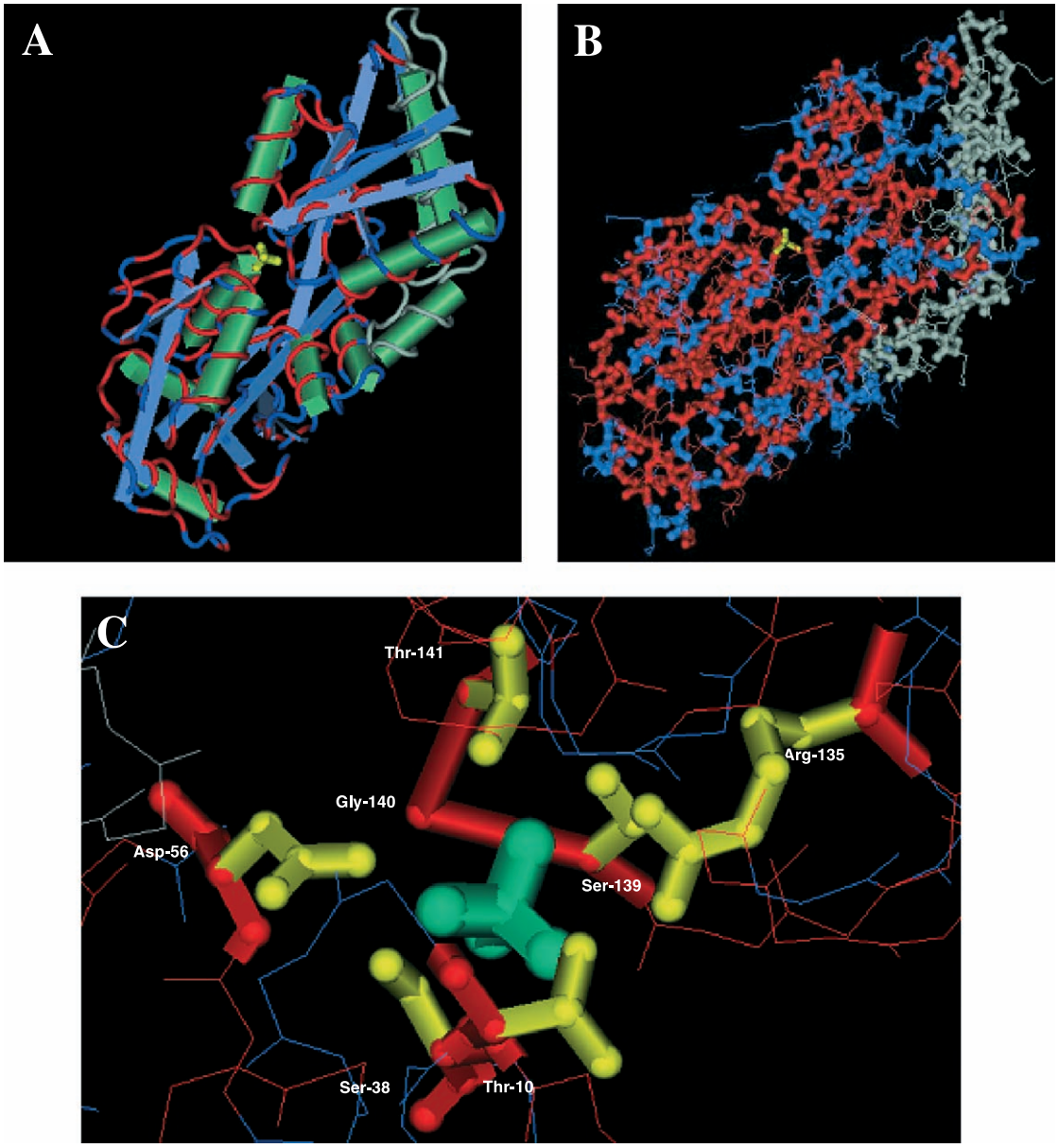

Fig. 4. Structural alignment between *E. coli* PstS and the *At. ferrooxidans PstS1* homolog. (A) secondary structure style; (B) hierarchical style. The identity between the two proteins is shown in red. The gray regions show a segment of PstS from *E. coli* that is not present in the protein from *At. ferrooxidans.* The phosphate molecule bound to its binding site is shown in yellow. (C) Structure of the phosphate binding site of PstS protein from *E. coli* (taken from Luecke and Quiocho, 1990) aligned with the putative equivalent site in the PstS-like protein from *At. ferrooxidans*. The amino acid residues interacting directly with the phosphate molecule are indicated and are represented by yellow tubes. The rest of the structure is shown as a wire for clarity. The phosphate molecule in green is seen bound to the binding site at the center of the figure.

Fig. 5 shows that the eight ORFs present in this region of the *At. ferrooxidans* DNA seem to be organized in two operons. The first one is the one composed of *phoB–phoR*, and the second is *pstSCAB–phoU–ppx*. This last operon shows almost the same physical organization as the *E. coli* operon

Table 1
Some properties of the proteins present in the Pho regulon from *E. coli* with the corresponding homolog proteins from *At. ferrooxidans*

Proteins	Molecular mass (kDa)		Theoretical isoelectric point		Identity (%)
	At. ferrooxidans	*E. coli*	*At. ferrooxidans*	*E. coli*	
PhoB	27.8	26.4	6.62	5.46	49
PhoR	49.7	49.6	5.44	9.63	37
PstS[a]	36.2	37	9.41	8.39	50
PstC	36.6	34.1	7.03	5.89	55
PstA	30.5	32.3	10.43	9.94	55
PstB	32.2	29	5.81	6.13	69
PhoU	26.4	27.4	5.03	5.14	44
PPX	53.4	58.1	7.01	6.65	34

[a] PstS1 from *At. ferrooxidans*.

(*pstSCAB–phoU*), except for the presence of the *ppx* gene. In *E. coli*, the *ppx* gene is located downstram of the *ppk* gene, both forming a separate operon. The Pho operon of *Burkholderia fungorum* LB400 deduced from the currently available NCBI genomic sequence (Chávez and Jerez, unpublished results) also shows similar features with the other Pho operons although with a different location for the *phoB*/*phoR* pair. The organization of the *ppx* and *ppk* genes is different in all the Pho regulon gene groupings shown in Fig. 5. *E. coli* has both *ppk* and *ppx* genes in the same operon (Kornberg et al., 1999), and therefore if they are coregulated at the transcriptional level, it is difficult to envisage an accumulation of polyP granules since the two enzymes have opposite activities (synthesis versus degradation of polyP). In fact, *E. coli* only transiently increases polyP synthesis under adverse conditions, and no polyP granules have been reported in this bacterium. One could speculate that microorganisms having the *ppk* and *ppx* genes organized in separate operons, could regulate synthesis and degradation of polyP separately, allowing them to accumulate these polymers in response to stressing conditions. Interestingly, we have observed that both *At. ferrooxidans* (Alvarez et al., 2001) and *B. fungorum* (Chávez and Jerez, unpublished results) have great capacity to accumulate polyphosphate granules. It will be of great interest to study the mechanism of regulation of the expression of these operons and the role they could play in adaptation of *At. ferrooxidans* to its environment.

In conclusion, we have shown, using 2D PAGE for protein separation and reverse genetics for gene determinations, that the putative phosphate and arsenate binding protein PstS1 present in *At. ferrooxidans* is highly conserved when compared with *E. coli* PstS both in sequence and structure. *At. ferrooxidans* ATCC 23270 contains a putative Pho regulon similar to the one present in other bacteria. This is also likely to occur in strain ATCC 19859, since the genes from this strain that we have sequenced so far (not shown)

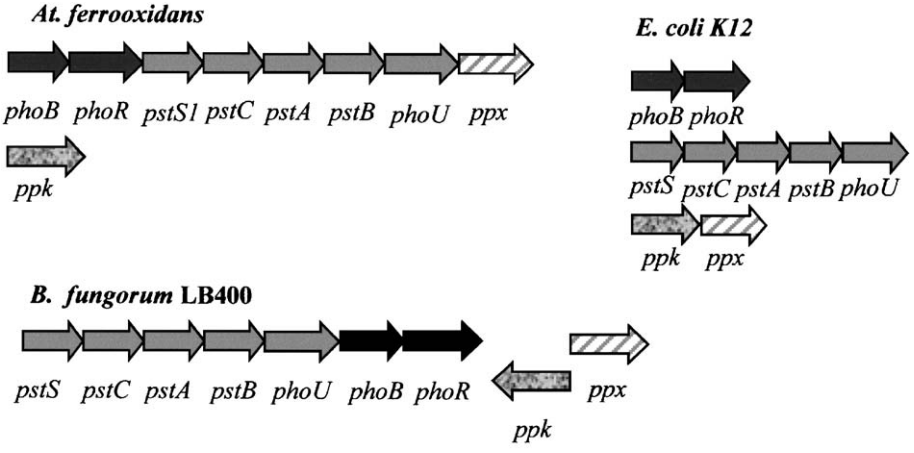

Fig. 5. Possible genetic organization of the ORFs flanking the putative *At. ferrooxidans pstS1* compared with Pho operons from other microorganisms. Arrows indicate the proposed direction and approximate extension of the putative ORFs.

have 98–99% identity compared with those of strain ATCC 23270. Obviously, it will be necessary to confirm the proposed genetic organization with expression studies.

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