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Periplasmic Proteins of the Extremophile *Acidithiobacillus ferrooxidans*:

A HIGH THROUGHPUT PROTEOMICS ANALYSIS*,S

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Abstract

Acidithiobacillus ferrooxidans is a chemolithoautotrophic acidophile capable of obtaining energy by oxidizing ferrous iron or sulfur compounds such as metal sulfides. Some of the proteins involved in these oxidations have been described as forming part of the periplasm of this extremophile. The detailed study of the periplasmic components constitutes an important area to understand the physiology and environmental interactions of microorganisms. Proteomics analysis of the periplasmic fraction of *A. ferrooxidans* ATCC 23270 was performed by using high resolution linear ion trap-FT MS. We identified a total of 131 proteins in the periplasm of the microorganism grown in thiosulfate. When possible, functional categories were assigned to the proteins: 13.8% were transport and binding proteins, 14.6% were several kinds of cell envelope proteins, 10.8% were involved in energy metabolism, 10% were related to protein fate and folding, 10% were proteins with unknown functions, and 26.1% were proteins without homologues in databases. These last proteins are most likely characteristic of *A. ferrooxidans* and may have important roles yet to be assigned. The majority of the periplasmic proteins from *A. ferrooxidans* were very basic compared with those of neutrophilic microorganisms such as *Escherichia coli*, suggesting a special adaptation of the chemolithoautotrophic bacterium to its very acidic environment. The high throughput proteomics approach used here not only helps to understand the physiology of this extreme acidophile but also offers an important contribution to

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the functional annotation for the available genomes of biomining microorganisms such as *A. ferrooxidans* for which no efficient genetic systems are available to disrupt genes by procedures such as homologous recombination.

Acidithiobacillus ferrooxidans is generally found in acidic environments such as mining dumps and acid mine drainages. It is a chemolithoautotrophic Gram-negative γ-proteobacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds (Refs. 1–3 and references therein). The ability of this and other microorganisms present in their habitat to solubilize metal sulfides has been successfully applied in biomining operations (1, 4).

The reactions involved in ferrous iron oxidation have been studied in detail (1, 5). The terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin and a cytochrome c_{552}. A high molecular weight *c*-type cytochrome, Cyc2, which is located in the outer membrane of *A. ferrooxidans*, has been suggested to be the prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (6). This pathway would be Cyc2 → rusticyanin→ Cyc1 (c_{552}) → aa_3 cytochrome oxidase (1).

The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (2, 7). On the other hand, thiosulfate has been postulated as a key intermediate compound in the oxidation of the sulfur moiety of pyrite (thiosulfate mechanism) (2). Sulfur compound-oxidizing enzymes such as thiosulfate-oxidizing enzyme in *A. ferrooxidans* (8) or tetrathionate hydrolase in *Acidithiobacillus thiooxidans* or *Thiobacillus ferrooxidans* (9, 10) may be involved in the process. Also a rhodanese activity has been described previously in *A. ferrooxidans* (11). This enzyme is a thiosulfate sulfurtransferase (TST),[1] which breaks the S–S bond present in thiosulfate, generating sulfur and sulfite. We have recently described in *A. ferrooxidans* several predicted cytoplasmic TST-like proteins and an exported TST-like protein (P21) that is highly expressed when the bacterium is grown in pyrite and sulfur but not in ferrous iron (12). The genomic contexts of some of the rhodanese-like genes suggested their implication in sulfur oxidation and metabolism, formation of Fe-S clusters, or detoxification mechanisms (13).

The most relevant reactions for ferrous iron and sulfur oxidation take place at the periplasmic space of *A. ferrooxidans* (1, 3). However, little is known about the periplasm of this acidophile and its components. To further study the proteins that may be involved in the oxidation of sulfur and metal sulfides, we analyzed and characterized by high throughput expression proteomics the proteins present in the periplasmic fraction of this bacterium.

[1]The abbreviations used are: TST, thiosulfate sulfurtransferase; ABC, ATP-binding cassette; OMP, outer membrane protein; LPS, lipopolysaccharide; PPIase, peptidyl-prolyl cis-trans isomerase; HiPIP, high redox potential iron-sulfur protein; Tat, twin arginine translocation; PQQ, pyrroloquinoline quinone; 2-D, two-dimensional; NEPHGE, non-equilibrium pH polyacrylamide gel electrophoresis; TIGR, The Institute for Genomic Research; CELLO, subcellular localization predictor; PENCE, Protein Engineering Network of Centres of Excellence; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; EF, elongation factor.

We found 131 proteins in the periplasmic fraction of the acidophilic *A. ferrooxidans* grown in thiosulfate. These include many transport and binding proteins, cell envelope proteins, energy metabolism proteins, and proteins involved in fate and folding. In addition, several proteins were identified as having unknown functions, and around 26.1% of them were proteins with no homologues in databases, many of which may be characteristic of this microorganism. The results presented not only contribute to understand the physiology of *A. ferrooxidans* but are also important for the genomic annotation of the new periplasmic proteins from this extremophile.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

A. ferrooxidans strain ATCC 23270 was grown in thiosulfate by using DSMZ medium 71 containing 20 mM thiosulfate and the following components: 3.0 g liter^{-1} KH_2PO_4, 0.5 g liter^{-1} $MgSO_4 \cdot 7H_2O$, 3.0 g liter^{-1} $(NH_4)_2SO_4$, 0.25 g liter^{-1} $CaCl_2 \cdot 2H_2O$. The pH was adjusted to about 4.6 by addition of 1 M NaOH.

Preparation of Periplasmic Fractions

To determine the most appropriate and efficient method to obtain the periplasmic proteins from *A. ferrooxidans*, we tested both the most commonly used osmotic shock procedure of Laundenbach *et al.* (14) and the chloroform-based method of Ames *et al.* (15). Possible cell lysis during the liberation of periplasmic proteins was controlled by determining the presence of an abundant cytoplasmic protein such as elongation factor (EF)-Tu by using Western blotting analysis with an antiserum against EF-Tu from *Escherichia coli*.

We found that the method of Laundenbach *et al.* (14) as applied to *A. ferrooxidans* released a high concentration of EF-Tu together with the periplasmic fraction, clearly indicating some cell disruption. On the other hand, when the chloroform method was used, no detectable EF-Tu was identified in the periplasmic preparations by using the immunological method (not shown). Furthermore the chloroform method gave a higher enrichment of rusticyanin, a well characterized periplasmic protein from *A. ferrooxidans*. Consequently the method of choice was that from Ames *et al.* (15) with minor modifications.

Briefly the harvested cell pellet (from a 200-ml culture at ~1×10^8 cells/ml) was washed twice with a basal salt solution (0.77 mM $(NH_4)_2SO_4$, 1.63 mM $MgSO_4$, 0.175 mM K_2HPO_4, pH 2.5) and was resuspended in 200 μl of the same acidic solution followed by the addition of 20 μl of chloroform. After briefly vortexing, the tubes were maintained at room temperature for about 15 min and adjusted to pH 2.5 by adding 10 mM Tris-HCl, pH 8. The cells were separated by centrifugation at 6,000 × *g* for 20 min, and the supernatant fraction containing the periplasmic proteins was withdrawn and brought to 100 μg/ml phenylmethylsulfonyl fluoride. Finally the proteins present in the periplasmic fraction were concentrated by vacuum to dryness in a vacuum concentrator (UniVapo 100H, Uniequip).

Two-dimensional (2-D) Non-equilibrium pH Polyacrylamide Gel Electrophoresis (NEPHGE) and SDS-PAGE

Total cell proteins were separated by 2-D NEPHGE (16) performed as described before for *A. ferrooxidans* (12, 17) using ampholytes (pH 3–10) from Bio-Rad. Cell samples (4 mg wet weight of cells) to be analyzed were resuspended in 80 μl of sonication buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 μg of pancreatic RNase/ml). Cell disruption was done by using a Misonix XL2020 sonicator with a microtip (six times during 30 s) followed by treatment with DNase (50 μg/ml final concentration). The mixture was then lyophilized. Total cell proteins obtained or the proteins from the periplasmic fraction were dissolved in lysis buffer and were analyzed by 12.5% SDS-PAGE, and the protein arrays obtained were visualized by silver staining as described before (17).

In-gel Trypsin Digestion

Lyophilized periplasmic protein pellets were solubilized directly in sample loading buffer, and an aliquot of the sample (~20 μg of proteins/lane) was separated on a 12.5% SDS-polyacrylamide gel. The gels were stained with Coomassie Blue R-250 for 18 h. The lanes containing the total periplasmic samples were cut evenly into five slices from the top to the bottom of the gel. In-gel trypsin digestion was performed using the procedure of Shevchenko *et al.* (18).

Liquid Chromatography-coupled Mass Spectrometric Analysis

An aliquot of the periplasmic protein digest was loaded onto a fused silica precolumn (outer diameter × inner diameter, 360 × 75 μm) packed with irregular C_{18} beads (5–20 μm, ODS-AQ, YMC, Waters, Milford, MA) and washed with 0.1% acetic acid. The precolumn was then Teflon sleeve-connected (outer diameter × inner diameter, 0.012 × 0.060 inch; Zeus, Orangerburg, SC) to a fused silica analytical column (outer diameter × inner diameter, 360 × 50 μm) packed with 5 cm of regular C_{18} beads (5 μm, ODS-AQ, YMC). An electrospray emitter tip (2–4 μm in diameter) was pulled with a laser on one end of this analytical column as described previously (19). An Agilent 1100 Series binary HPLC system (Palo Alto, CA) was interfaced with a hybrid linear ion trap (LTQ)-FT mass spectrometer (ThermoElectron, San Jose, CA) equipped with a microelectrospray source for on-line peptide separations. The instrument was operated in the data-dependent mode using Xcalibur software (ThermoElectron). It cycled through a single MS experiment using the FT-ICR cell as the mass analyzer (resolution, R = 25,000 at m/z 400; target value, 1×10^6) followed by 10 MS/MS experiments using the linear ion trap as the mass analyzer. The dynamic exclusion duration was set to be 45 s. The HPLC gradient used for the sample analysis was: 0–7% B in 5 min, 7–45% B in 70 min, 45–100% in 15 min, and 100–0% B in 5 min (A = 0.1 M acetic acid (Sigma-Aldrich), B = 70% acetonitrile (Mallinckrodt, Paris, KY) in 0.1 M acetic acid).

Data Analysis

When we started this study, the available *A. ferrooxidans* genome (The Institute for Genomic Research (TIGR), www.tigr.org) was not yet annotated. Therefore, we used the GLIMMER (Gene Locator and Interpolated Markov Modeler, version 2.10) software tools to predict protein-encoding ORFs within the available *A. ferrooxidans* genomic sequence

and hence constructed our local putative *A. ferrooxidans* protein database to be used in the MS/MS proteomics analysis (20, 21). Briefly long ORFs were extracted from the genomic contigs with "long-orfs(1)" and used to train an interpolated Markov model with "build-icm." The resulting model was used to scan the genomic contigs for high scoring ORFs with "glimmer2(1)," yielding 3,727 non-overlapping ORF predictions. Each ORF or putative protein was provisionally annotated by identifying the highest scoring homologous protein from a Smith-Waterman search of the National Center for Biotechnology Information (NCBI) nonredundant protein database (downloaded July 27, 2003) (22), requiring a statistical expectation of 1*e*–6 or better. The acquired MS/MS data were then searched against the putative *A. ferrooxidans* protein database using SEQUEST (23). SEQUEST search parameters were conducted with "no enzyme" specificity and a static modification of 57 amu on cysteine, representing alkylation with iodoacetamide, and a differential modification of 16 amu on methionine, representing the possibility of oxidation. The parent ion mass window was set as ±0.05 Da (monoisotopic mass), and the fragment ion mass tolerance was set as ±0.35 Da (monoisotopic mass). The parameters DelMass < 1.0, Xcorr > 2.4, DelCn > 0.1, Sp > 500, RSp < 10, and Ion Ratio > 0.6 were used to evaluate database search results, and the reported peptide sequences were manually validated (24). Detailed additional information for all annotated proteins with predicted export signals identified in the periplasmic fraction can be seen in Supplemental Table S1. For protein identifications based on single peptide assignments, the sequences of the peptides used to make each assignment, the precursor masses and charges observed, and the scores for each of these peptides are shown in Supplemental Table S2, and their corresponding spectra are shown in Supplemental Fig. S1.

Sequence Analysis

Identity/similarity and conserved domain searching in databases was done by using the BlastP program (25) and Conserved Domain Database from NCBI (www.ncbi.nlm.nih.gov). Because we started using the finished non-annotated available *A. ferrooxidans* ATCC 23270 genomic sequence (www.tigr.org), we determined the ORFs for all the periplasmic proteins found. Now that the TIGR final annotation is available, we compared both databases and have indicated the minor discrepancies when pertinent. Molecular masses and isoelectric points of ORFs were obtained by using Compute pI/Mw tool (www.expasy.org/tools/pi_tool.html). The presence of export signals for each protein found in the periplasmic fraction was predicted by using SignalP (www.cbs.dtu.dk/services/SignalP), TatP (www.cbs.dtu.dk/services/TatP-1.0), SecretomeP (www.cbs.dtu.dk/services/SecretomeP), and LipoP (www.cbs.dtu.dk/services/LipoP). The predicted subcellular locations of the proteins coded by the different ORFs analyzed were obtained using PENCE Proteome Analyst (www.cs.ualberta.ca/%7Ebioinfo/PA/Sub/index.html) and CELLO (cello.life.nctu.edu.tw) programs. The functional categories for the different identified proteins were obtained from the genomic sequence of *A. ferrooxidans* (www.tigr.org).

RESULTS AND DISCUSSION

General Characteristics of the Periplasmic Proteins from A. ferrooxidans

A. ferrooxidans cells were obtained by growing the bacteria in thiosulfate as described under "Experimental Procedures." The periplasmic fraction was prepared and checked by SDS-PAGE and 2-D PAGE as shown in Fig. 1. As expected, the periplasmic fraction (Fig. 1*B*) contained a reduced number of proteins compared with the total cellular proteins (Fig. 1*A*). Furthermore many of the most abundant proteins present in the total fraction were absent in the periplasmic preparation. These results together with the absence of EF-Tu in the periplasmic fraction (see "Experimental Procedures") indicate a low amount of cell lysis during the preparation of this fraction. Fig. 1*C* shows the periplasmic fraction proteins separated in a monodimensional SDS gel from which the five sections from top to bottom of the gel were obtained (see "Experimental Procedures"). A total of 131 proteins were identified from the *A. ferrooxidans* periplasmic sample using tandem mass spectrometry. The proteins identified were first categorized according to their predicted export signals as illustrated in Fig. 2*A*. Most of the proteins (62.3%) possessed export signals of the Sec type. Five of them (3.8%) showed a twin arginine translocation (Tat) signal, suggesting that they would be proteins exported in a folded form across the cytoplasmic membrane by the Tat pathway. Of the remaining proteins, 11.5% could be considered as possibly periplasmic according to the SecretomeP program, 8.5% could be exported according to their subcellular location prediction (CELLO and PENCE Proteome Analyst), and 13.8% of the proteins found in the periplasmic fraction did not contain any predicted export signal. These last putative proteins were expected to have a cytoplasmic localization. However, it is known that some proteins are exported without having typical export signals (26). Therefore, some of them could be exported, although others could be the result of some degree of cell lysis during the preparation of the periplasmic fraction. The identified 131 periplasmic proteins with typical export signals were further grouped according to functional categories as shown in Fig. 2*B* and Table I. Fig. 3*A* shows the distribution of the *A. ferrooxidans* periplasmic proteins according to their molecular weights and their corresponding calculated isoelectric points. The majority (70.8%) of the periplasmic proteins of this acidophilic microorganism showed pI values higher than 7, and only 29.2% showed pI values below pH 7. When the results obtained for the periplasmic proteins of *A. ferrooxidans* were compared with those obtained experimentally for the periplasmic proteins from *E. coli* (27) and *Synechocystis* sp. (28) a different distribution of the pI values for the proteins was observed. As predicted in Fig. 3*B* using SignalP or TatP, pI distribution of proteins in neutrophiles *E. coli*, *Thiobacillus denitrificans*, and *Geobacter sulfurreducens* are comparable. They differ drastically from the pI distributions of the acidophilic *A. ferrooxidans* and *Coxiella burnetii* or the acid tolerant *Helicobacter pylori*. These acid-resistant microorganisms have much higher percentages of basic proteins in their periplasms. On the contrary, the bioinformatics analysis of alkalophiles such as the *δ*-proteobacterium MLMS-1 and *Alkalilimnicola ehrlichii* showed a much higher percentage of acidic proteins. The great amount of highly basic proteins in the periplasm of *A. ferrooxidans* (16.8% of the proteins with pI higher than 10) revealed in this study a special adaptation of this acidophile to its acidic environment. It has been reported that the pH of its periplasm is in the 2.5–3.0 range (5). Most of these proteins are therefore likely acid-tolerant and/or acid-stable. One example is rusticyanin, an

abundant redox blue copper protein with a pI of 8.85 that is typically purified by acidifying the *A. ferrooxidans* extract to pH 2–4 (29).

In a bacterium such as *H. pylori*, both the inner and outer membrane of the organism can be exposed to high acidity. It has been argued that because most of these proteins have an isoelectric point significantly more alkaline than that of neutrophiles (30), they would retard the flux of protons into the periplasmic space. A similar situation may take place for several of the *A. ferrooxidans* exposed proteins. Accordingly we estimated before that all the external putative loops of the major outer membrane protein Omp40 from *A. ferrooxidans* would have a positive net charge at pH 2.5 (31). Being positively charged, the pore would restrict the diffusion of protons from both outside and the periplasmic space toward the environment. These modifications may be a general functional adaptation of acidophiles that provides them with a degree of resistance to the acid present in their environment.

As shown in Fig. 2*B* and Table I, periplasmic proteins identified were further grouped according to functional categories. Almost 50% of the periplasmic proteins belonged to the cell envelope, protein fate, transport and binding proteins, and energy metabolism categories. On the other hand, about 36% of the proteins comprised proteins with no homologues in databases, being proteins with unknown functions that most likely are unique to *A. ferrooxidans*. Based on what is known or has been proposed for other microorganisms, we show schematically in Fig. 4 the possible locations and in some cases the suggested functions of the proteins present in the periphery of *A. ferrooxidans.*

Transport and Binding Proteins

A. ferrooxidans contains two *pstS* genes, both of which form part of an apparent Pho regulon (32) because both are induced when *A. ferrooxidans* is grown in the absence of phosphate and may represent an adaptation of the microorganism for the survival under scarce phosphate availability. AFE_1151 and AFE_1648 in Table I corresponded to the genes coding for phosphate-binding proteins PstS2 and PstS1, respectively (Fig. 4), which may form part of ABC-type periplasmic phosphate transporters in *A. ferrooxidans*.

TonB-dependent receptors are outer membrane proteins that form channels permeable to large solutes such as vitamin B_{12} and siderophores. These receptors bind ligands with high affinity and require the interaction with the TonB protein (Ref. 33 and references therein). In *A. ferrooxidans*, AFE_0174, AFE_1054, and AFE_3103 are putative TonB-dependent receptors (Fig. 4) and may have a function equivalent to those from *E. coli*. We also identified in the periplasm another protein (AFE_0169) that showed only a conserved TonB-dependent receptor domain.

AFE_1590 has been annotated as a putative periplasmic iron compound-binding protein that belongs to the TroA superfamily. Bioinformatics analysis of the *A. ferrooxidans* genome showed 11 putative genes coding for siderophore outer membrane receptors, all grouped in seven gene clusters (34). According to these authors, AFE_1590 is located at “cluster B,” which potentially encodes for a complete suite of proteins needed for Fe(III) uptake and also has an upstream putative fur box. The identification and presence of the periplasmic binding protein TroA (AFE_1590) (Fig. 4) in the periplasmic fraction from *A. ferrooxidans* grown in

thiosulfate strongly suggests that this gene cluster is used in *A. ferrooxidans* for Fe(III) uptake under this growth condition.

AFE_2463 is a putative protein with similarity to TolC, a common component of a wide variety of efflux pumps (see Fig. 4). TolC and similar proteins are involved in the export of chemically diverse molecules ranging from large protein toxins such as α-hemolysin to small toxic compounds such as antibiotics (33).

The Tol system is involved in outer membrane stability by establishing the structural network linking outer membrane to the peptidoglycan, driving newly synthesized outer membrane components across the periplasm, and the surface expression of *O*-lipopolysaccharide (35). AFE_2957 was found to be similar to TolB, the periplasmic component of the Tol biopolymer transport system. Candidate genes coding for the other components of the Tol-Pal system (TolAQR) were also present in the genetic context of AFE_2957, but we did not find them in our periplasmic fraction.

Fig. 4 also shows the location for AFE_2438, which is a putative periplasmic cation-binding protein of the TroA family similar to the periplasmic zinc-binding protein ZnuA. Downstream in the genetic context of AFE_2438 there are genes coding for proteins with similarities to the high affinity zinc ABC-type transporter: the ATP-binding protein (ZnuC) (AFE_2437) and a duplicated permease (ZnuB) (AFE_2436 and AFE_2435) that could form part of one transcriptional unit (36).

AFE_2865 and AFE_1256 are periplasmic putative toluene tolerance proteins (Ttg2D) (Fig. 4). The *ttg2D* gene encodes a periplasmic component of an ABC-type transport system involved in resistance to organic solvents (37). The surrounding three ORFs in this *A. ferrooxidans* putative cluster (AFE_2866, AFE_2867, and AFE_2868) have similarity to genes *ttg2ABC*, which are part of the *Pseudomonas putida* operon involved in toluene resistance (37). It is expected that toluene resistance would be related to extrusion of toluene; however, the operon in *P. putida* has been shown to be a four-member system typical of the ABC uptake family systems. In addition, the whole-genome analysis of transporters in the plant pathogen *Xylella fastidiosa* suggests that a *ttg* putative operon may be related to uptake of glutamate and other polar amino acids instead of being a system for toluene resistance (38). In fact, proteins with homology to Ttg proteins of *P. putida* were described in *Neisseria meningitidis*, and their participation in the uptake of L-glutamate was verified (39). Interestingly it has recently been found that despite being an obligate autotroph *A. ferrooxidans* can use exogenous glutamate[2] suggesting the existence of a possibly functional glutamate transporter in this microorganism.

AFE_1586 (ModA3) is the candidate gene for the periplasmic component of an ABC-type molybdate transport system (Fig. 4) that belongs to the ModA family (40). Upstream of the genetic context of AFE_1586 from *A. ferrooxidans* there is a hypothetical protein (AFE_1587) and a cation diffusion facilitator family transporter (AFE_1588) suggesting a role for AFE_1586 in cation transport. Two other putative periplasmic components of an

[2]O. Orellana, personal communication.

ABC-type molybdate transporter system were found in the periplasmic fraction: AFE_2981 or ModA1 and AFE_2975 or ModA2 (Fig. 2). These transport systems allow both uptake and efflux and have different ion specificities. Structural modeling of ModA proteins with crystal structures of known similar proteins strongly suggests a functional and conserved mechanism for the transport of thiosulfate/sulfate or molybdate in *A. ferrooxidans* (41). By expression proteomics we found previously that ModA1 and ModA2 are synthesized in much higher amounts in cells grown in sulfur or thiosulfate as compared with those grown in ferrous iron (17, 41). The almost absent expression of these putative binding proteins in ferrous iron suggests that these proteins are related with sulfur compound metabolism in this acidophile (Fig. 4). Recently in a proteomics study of *A. ferrooxidans* total proteins, Bouchal *et al.* (42) described a sulfate/molybdate-binding protein expressed in high abundance during sulfur growth condition. These authors suggested that in all probability this protein is the same gene product we previously annotated as ModA1 (13, 17).

In the “central intermediary metabolism: sulfur metabolism category” we found that AFE_2979 (Table I) corresponded to the periplasmic sulfur-pyrite-thiosulfate-sulfide-induced protein P21 that we have described before (12). The genomic context of the rhodanese-like gene *p21* contains the gene *modA1* and *modA2* and the two putative thiosulfate quinone oxidoreductases (*doxDA1* and *doxDA2*) (3, 13). These genes were all highly expressed as determined by real time PCR and DNA macroarray studies in cells grown in thiosulfate compared with the levels seen in ferrous iron (12, 13, 17). Furthermore we have described in *A. ferrooxidans* that the transcription levels of *doxDA1* and *doxDA2* genes are induced when the microorganism was grown in pyrite compared with ferrous iron growth.[3]

Cell Envelope

Outer membrane proteins (OMPs) have been identified as adhesins, architectural proteins, passive diffusion pores, siderophore receptors, efflux channels, protein translocation pores, and enzymes (*e.g.* lipases, proteases, and palmitoyltransferases) (33). OMPs are synthesized in the cytoplasm and are translocated across the inner membrane. Passage through the periplasm presents a number of challenges due to the hydrophobic nature of the OMPs, the acidic pH of the periplasm in the case of *A. ferrooxidans*, and the choice of membranes into which they can insert (43). Most likely, the periplasmic intermediates of the OMPs diffuse through the periplasm before they are inserted into the outer membrane (44). In this regard, Eppens *et al.* (45) demonstrated that PhoE passes through the periplasm on its way to the outer membrane. It is also known that misfolded OMPs are present in the periplasm of *E. coli* under stressing conditions (46). Several possible outer membrane precursor proteins were found in the periplasmic fraction from *A. ferrooxidans* analyzed in Table I. It is possible that these intermediates or possibly misfolded envelope proteins were detected in our case due to the high dynamic range and specificity of the FT MS that allows their recognition.

[3]Valenzuela, L., Chi, A., Beard, S., Shabanowitz, J., Hunt, D. F., and Jerez, C. A. (2007) Differential-expression proteomics for the study of sulfur metabolism in the chemolithoautophic *Acidithiobacillus ferrooxidans* in *Microbial Sulfur Metabolism* (Friedrich, C. G., and Dahl, C., eds), pp. 77–86, Springer Press, Berlin.

AFE_0848 showed identity to a carbohydrate-selective porin of the OprB family (Fig. 4). The OprB porin from *P. aeruginosa* is permeable to solutes of <300 kDa but shows specificity for monosaccharides (33).

AFE_2654 showed similarity to an outer membrane protein of the OMPP1/FadL/TodX family involved in translocation of long-chain fatty acids across the outer membrane and in alkyl-aromatic compound catabolism (47). Downstream of the genomic context of AFE_2654 is AFE_2655, a candidate gene for a LolA protein that was detected in the periplasmic fraction (Fig. 4). AFE_2655 did not show a typical export signal but was predicted to be located in the periplasm by the PENCE Proteome Analyst and CELLO programs. LolA is a periplasmic chaperone involved in lipoprotein transport through the bacterial cell envelope (48). After transport via the Sec system, lipoproteins bind to the ABC transporter LolCDE. Energy from ATP hydrolysis by LolD is transferred to LolC and LolD and then used to open the hydrophobic LolA cavity to accommodate the lipoprotein. When the LolA-lipoprotein complex interacts with the outer membrane receptor LolB, the lipoprotein is transferred to LolB and then inserted into the outer membrane (48) (Fig. 4). Candidate genes for LolCD (AFE_2555 and AFE_2556) and LolB (AFE_0343) were also present in the genome sequence from *A. ferrooxidans* ATCC 23270 (www.tigr.org).

Fig. 4 indicates a possible location for the protein coded by AFE_0560 that also belongs to the OMPP1/FadL/TodX outer membrane protein transport protein family. This protein was identified previously in our laboratory by 2-D PAGE as a sulfur-induced protein called Omp44 (17). *A. ferrooxidans* contained in its periplasmic fraction proteins identified as candidates SurA (AFE_0075) and Omp85 (AFE_1632) (Table I). Members of the Omp85 family are involved in the assembly of outer membrane proteins, LPS, glycolipids, and phospholipids (49). Most likely then outer membrane proteins from *A. ferrooxidans* are assembled and inserted into the outer membrane according to the currently proposed model for Gram-negative bacteria. The presence of LolA in the periplasmic fraction and of genes coding for putative LolB and LolCD in *A. ferrooxidans* strongly suggests that in this acidophilic microorganism the mechanism for the transport of lipoproteins from the inner membrane to the outer membrane is similar to that proposed for *E. coli* (48) (Fig. 4).

AFE_2579 showed similarity to a rare lipoprotein homologue of RlpB from *E. coli*. Recently it was reported that *r1pB* is an essential gene in *E. coli* that forms a complex with Imp, an important protein for outer membrane biogenesis, and functions in LPS assembly (50). In the genome of *A. ferrooxidans* there is a candidate gene for an Imp protein codified in a different genetic region (AFE_0076) that could function together with RlpB in LPS assembly in *A. ferrooxidans* (Fig. 4).

Fig. 4 also shows the location for the proteins coded in the AFE_0783 and AFE_2748. AFE_0783 is a candidate gene for an outer membrane lipoprotein of the Slp family that in *E. coli* is known to be an outer membrane lipoprotein (43). AFE_2748 is a putative gene for an outer membrane lipoprotein of the SmpA/OmlA family that is possibly involved in maintaining the structural integrity of the cell envelope.

AFE_2953 and AFE_2722 are peptidoglycan-associated lipoproteins of the OmpA family (Fig. 4). As in the case of OmpA, they could have a role in maintaining the bacterial surface integrity acting as a physical linkage between the outer membrane and the peptidoglycan (51). AFE_2722 showed similarity to an OmpA-like outer membrane protein, FopA (Fig. 4), which was identified previously as an NaCl-regulated major outer membrane proteins of *A. ferrooxidans* strain NASF-1.[4]

We also found in the periplasmic fraction AFE_2087 that is similar to the osmoregulated periplasmic glucan biosynthesis protein MdoG (52) and AFE_0158, AFE_0297, AFE_0745, AFE_0850, and AFE_2445 that were identified as putative lipoproteins of unknown function (Fig. 4).

AFE_2621 (not shown in Fig. 4) has a short N-terminal sequence that directs the methylation of a conserved phenylalanine residue (Pfam *N*-methyl, PF07963) often found at the N terminus of pilins and other proteins involved in secretion. However, the periplasmic protein encoded by AFE_2621 appears to be unique for *A. ferrooxidans*. AFE_3018 (not included in Fig. 4) was found to be similar to a putative pili assembly chaperone, transmembrane protein of the FimC and PapD family (Table I). PapD-like chaperones bind, stabilize, and cap interactive surfaces of subunits until they are assembled into the pilus (53). The presence of fimbria and pili-like appendages has been reported previously for *A. ferrooxidans* (54).

Protein Fate

A range of periplasmic proteins were identified and implicated in the targeting and assembly of extracytoplasmic proteins and as such are potential candidates as facilitators for OMP biogenesis. These folding factors include molecular chaperones, protein folding catalysts, and proteases. Two types of folding catalysts are found in the periplasm, namely protein-disulfide isomerases and peptidyl-prolyl cis-trans isomerases (PPIases) (43). We found two putative PPIases in the periplasm of *A. ferrooxidans* (AFE_0075 and AFE_2516) (Table I and Fig. 4). PPIases have been shown to facilitate the cis-trans isomerization of proline residues both *in vitro* and *in vivo*. AFE_0075 is a protein with similarity to *E. coli* SurA, a periplasmic protein that possesses both chaperone and PPIase activity. AFE_2516 is a protein with a PPIase domain, but it did not show similarity to known proteins. For this putative protein we can infer a function similar to that of SurA and other PPIases (55).

Periplasmic and outer membrane proteins often contain disulfide bonds. Their formation is facilitated *in vivo* by a number of specialized thiol-disulfide exchange enzymes, which are involved in the formation, isomerization, and reduction of disulfide bonds. DsbC and DsbG are homodimeric disulfide isomerases that resolve incorrectly formed disulfide bonds. They are maintained in a reduced state by DsbD, which is regenerated by the cytoplasmic thioredoxin reductase system (56). We found one DsbD homologue (AFE_2493) (Fig. 4), a protein without a predicted export signal (not shown in Table I), and one DsbG homologue (AFE_162) in the periplasm, but we did not find proteins with similarities to DsbC protein

[4]K. Kamikura, unpublished results.

in the complete genome of *A. ferrooxidans*. However, we found another protein in the periplasmic fraction (AFE_0851) with a DsbG domain that possesses an active site similar to the active site of SoxW, a thioredoxin involved in the transfer of electrons to an unknown periplasmic target of the thiosulfate-oxidizing enzyme during lithotrophic growth of *Paracoccus pantotrophus* in thiosulfate (57).

AFE_0259 and AFE_1685 (Table I) were found to be similar to two putative periplasmic serine proteases of the DO-DeqQ family. They are trypsin-like serine proteases, typically periplasmic, that contain a C-terminal PDZ domain. These serine proteases belong to the HtrA family of proteins (Fig. 4). In *E. coli*, DegP, DegQ, and DegS comprise the HtrA family of proteins. These three proteins exhibit a high degree of sequence similarity in their protease domains. However, DegP and DegQ harbor two PDZ domains (like AFE_0259 and AFE_1685), whereas DegS contains only one (58). In contrast to the high sequence similarity found in HtrA homologues of various species, one specific region in the protease domain exhibits only minimal sequence identity. This region of DegP is called the Q-linker as many Gln residues have been discovered in DegP of *E. coli* (59). Upon comparison of the sequences found near the Q-linker in AFE_0259 and AFE_1685, it was clear that both exhibited sequence similarity to the Q-linker of *E. coli* DegP, which is of a similar length. Therefore, we propose that the HtrA-like proteins annotated in *A. ferrooxidans* (www.tigr.org) should be renamed as DegP-like proteins (Fig. 4).

Energy Metabolism

In the electron transfer subgroup of the “energy metabolism functional category” (Table I), which is a key subgroup for the substrate oxidation ability of *A. ferrooxidans*, we detected and identified six proteins. All of them showed signal peptides, and the only protein showing a Tat signal in this group was the putative high redox potential iron-sulfur protein (HiPIP) or iron oxidase (Iro, AFE_0373). The HiPIP protein has been characterized previously in *A. ferrooxidans* (60, 61). This protein was proposed to be the first electron acceptor in several alternative models of the electron transfer chain between Fe(II) and oxygen (60, 62). However, recently Bruscella *et al.* (63) suggested that Iro is involved in an electron transfer chain between a cytochrome bc_1 complex functioning in the forward direction and a terminal oxidase as it occurs in other known HiPIPs (64). Bruscella *et al.* (63) cloned the gene coding for *A. ferrooxidans* HiPIP and overproduced it in the periplasm of *E. coli*. Translocation of this protein in the periplasm of *E. coli* was dependent on the *tatC* gene, indicating that the *A. ferrooxidans* HiPIP protein is dependent on the Tat system. Our results demonstrate indeed that this protein is present in the periplasm of *A. ferrooxidans* (Fig. 4).

The other five proteins with Sec export signals were cytochromes c_4 (CycA-1, AFE_0008 and CycA-2, AFE_0378), cytochrome *c* (Cyc2, AFE_3179), cytochrome c_{552} (Cyc1, AFE_3180), and rusticyanin (AFE_3186). All these redox proteins have been described to be located in the periplasm of *A. ferrooxidans* (1, 5, 17), and it has been confirmed here by our mass spectrometric analysis (Fig. 4). It is known that *cycA1* is codified by the *petI* operon and the *cycA2* is in *petII* operon (65). Very recently, a model has been presented in which *petI* was proposed to encode the bc_1 complex, functioning in the uphill flow of

electrons from iron to NAD(P), whereas *petII* was suggested to be involved in electron transfer from sulfur to oxygen (66). The participation of these two c_4 cytochromes in thiosulfate oxidation has not been reported previously. However, considering thiosulfate is a principal intermediary in pyrite (FeS_2) oxidation, these two c_4 cytochromes most likely have an important function during the mineral oxidation.

AFE_2996 has a pyrroloquinoline quinone (PQQ) enzyme repeat that has been found in several enzymes that utilize pyrroloquinoline quinone as a prosthetic group (67). This protein was previously reported as a sulfur-induced outer membrane protein in *A. ferrooxidans* MSR, and the corresponding gene was cloned and sequenced (68). This sulfur-induced outer membrane protein was recently annotated (EMBL-EBI accession number AB259312) as a tetrathionate hydrolase, and we have tentatively included it in Fig. 4. AFE_0847 and AFE_1154 (Table I and Fig. 4) were also annotated as PQQ enzyme repeat domain proteins.

AFE_0555 was a candidate gene for the lipoate-binding protein H of the glycine cleavage system. Lipoate acts as a swinging arm in the glycine cleavage system and 2-oxo acid dehydrogenase complex moving the substrate to the different active sites of the complex (69). Putative genes nearby are similar to genes related to lipoate metabolism and a putative heterodisulfide reductase complex. The function of these genes is entirely unknown in *A. ferrooxidans*.

Cellular Processes and Unknown Proteins with General Putative Functions

AFE_2607 (Table I) is a candidate gene for ComL similar to that of *Neisseria gonorrhoeae* (70). Although no information regarding this putative protein is currently available in *A. ferrooxidans*, we have tentatively included it in Fig. 4. AFE_0831 showed similarity to an acid phosphatase from *Methylococcus capsulatus*, and AFE_2266 has an incomplete purine nucleoside permease (NUP) domain, which is present in both bacteria and fungi (71). The function and specificity of both putative proteins (Fig. 4) are unknown in *A. ferrooxidans*.

HypA1 (AFE_2974) and HypA2 (AFE_2982) are export proteins, with no homologues in databases, that contain a Tat signal peptide (Fig. 4). Downstream of AFE_2982 in the genome of *A. ferrooxidans* are codified P21 and ModA1 proteins (12, 13). AFE_1396 is the carboxysome structural protein CsoS2 of *A. ferrooxidans*. This protein also contains a Tat signal peptide and forms a putative operon with the second copy of the Rubisco genes (*cbbL2* and *cbbS2*) (72).

Ribosomal Proteins

Table I shows the presence of three putative ribosomal proteins (S19, L11, and L35). These proteins showed a predicted SecretomeP export signal. This could be an indication that the predicting program gives some false positive hits in its predictions because ribosomal proteins would be expected to be located at the cytoplasm. Alternatively the exportation of these proteins may have an interesting but not yet defined role in this microorganism. However, it is also possible that these proteins were present in the periplasmic fraction due to cell lysis contamination.

Unknown Proteins

We found 28 proteins with export signals that were currently annotated as conserved hypothetical proteins, proteins containing domains of hypothetical proteins, or hypothetical proteins (Table II).

The proteins in Table II are most likely characteristic of *A. ferrooxidans* and may have important functions yet to be defined. Therefore, they should be reannotated as periplasmic proteins with unknown functions. The high throughput proteomics approach used here offers an important contribution to the functional annotation for the available genomes of acidophilic biomining microorganisms such as *A. ferrooxidans* for which no genetic systems are available to perform functional analysis by homologous recombination and genetic disruption of the genes of interest.

Non-annotated Proteins

All ORFs coding for the proteins shown in Tables I and II were searched against local synthetic databases before the *A. ferrooxidans* genome annotation was made available (www.tigr.org). The majority of them coincided with the TIGR annotation. However, we did identify a number of proteins in the periplasmic fraction that are not currently included in the TIGR database. This includes four proteins with no homologues in the databases, a putative sterol carrier protein, and a class 1 cytochrome *c*. We also experimentally confirmed a putative periplasmic copper resistance protein, CopC. The transcription of its gene has been validated by Northern electrophoresis,[5] thus supporting a possible functional role for it in *A. ferrooxidans*.

Proteins without Predicted Export Signals and Present in the Periplasmic Fraction

We also detected 30 proteins without export signals in the periplasmic fraction of *A. ferrooxidans* (not shown). Most of these proteins could be attributed to cell lysis during the preparation of the periplasm due to the detection of some abundant cytoplasmic proteins such as EF-Tu, carboxysome shell components, subunits of ribulose-bisphosphate carboxylase, and some ribosomal proteins (L17 and L7–L12). However, it is not clear why only some of the ribosomal proteins were present. Interestingly chaperone DnaK, the chaperonin GroEl, superoxide dismutase, EF-Tu, and some ribosomal proteins have also been found as non-classically secreted proteins mainly in Gram-positive bacteria (26). Therefore, their cellular locations would have to be further verified. Another protein without a typical export signal but found in the periplasm was AFE_2655, a protein with high similarity to LolA (Fig. 4). LolA was predicted to be periplasmically located when using PENCE Proteome Analyst and CELLO programs. It is therefore possible that in *A. ferrooxidans* its LolA-like protein is exported to the periplasm in a non-classical form.

Final Remarks

Reported here is the first detailed proteomics analysis of periplasmic proteins of *A. ferrooxidans*. The data gathered from this important biomining microorganism will (i) be a

[5]C. Navarro and C. A. Jerez, unpublished results.

valuable contribution to complete the annotation of *A. ferrooxidans* and future microbial genomes; (ii) aid in understanding the physiology of this acidophilic bacterium and its adaptation to its very acidic environment with high toxic metals concentrations; (iii) provide information on several unknown periplasmic proteins, most likely characteristic of *A. ferrooxidans* and that may have important roles in its functioning; and (iv) be the basis for further differential proteomics studies to determine the interactions of *A. ferrooxidans* peripheric components with its oxidizable substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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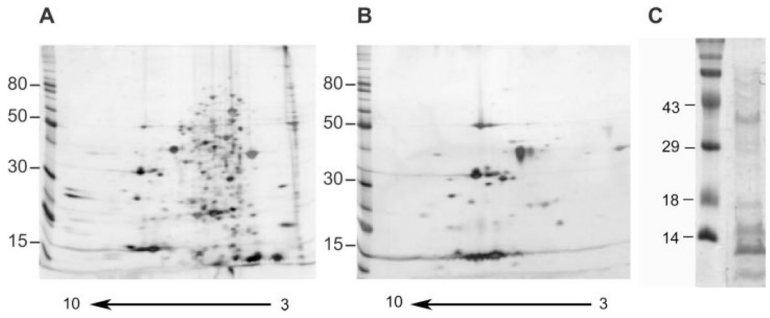

Fig. 1. 2-D PAGE of the periplasmic fraction of *A. ferrooxidans* grown on thiosulfate

Total proteins (*A*) or periplasmic fraction (*B* and *C*) were separated by 2-D NEPHGE (*A* and *B*) with a pH gradient between 3.0 (*right side* of the gel) and 10.0 (*left side* of the gel) or by monodimensional SDS-PAGE (*C*). Spots were detected by silver stain (*A* and *B*) or by colloidal Coomassie Blue (*C*). Molecular mass standards (in kilodaltons) are given on the *left* of the gels.

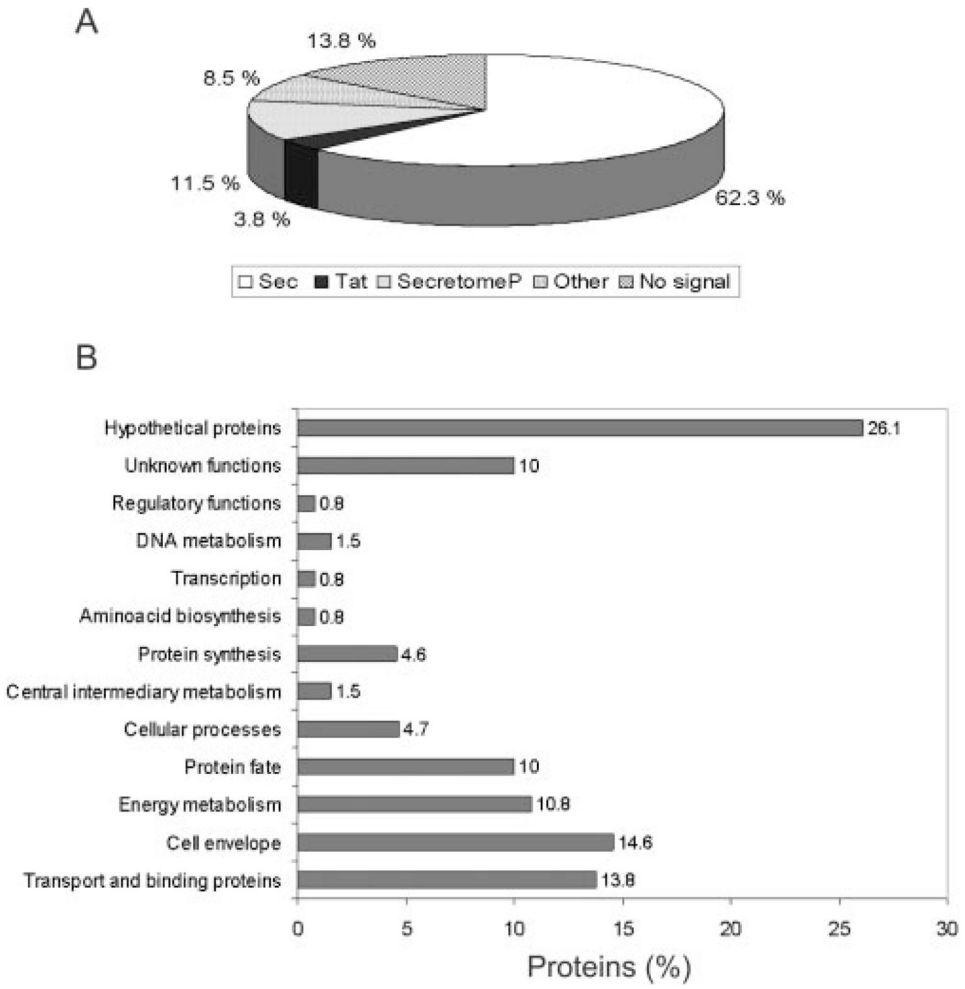

Fig. 2. Global analysis of periplasmic proteins from *A. ferrooxidans*

A, percent distribution of proteins according to their export signals. *B*, distribution of periplasmic proteins in functional categories according to TIGR annotation.

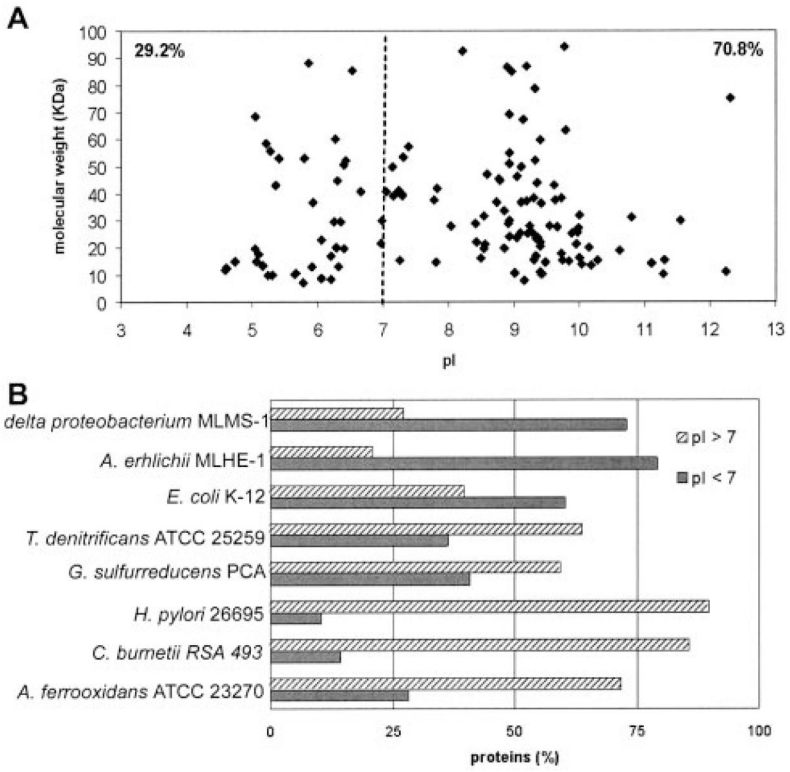

Fig. 3. pI distribution among periplasmic proteins found in *A. ferrooxidans* compared with some other Gram-negative micro-organisms

A, distribution of all proteins found in the periplasmic fraction from *A. ferrooxidans* ATCC 23270 grown in thiosulfate according to their theoretical molecular masses and isoelectric points. *B*, percentage of SignalP or TatP proteins with pI values above and below 7.0 predicted in complete genomes of Gram-negative bacteria. Alkalophiles, *δ*-proteobacterium MLMS-1 and *A. ehrlichii* MLHE-1; neutrophiles, *E. coli* K-12, *T. denitrificans* ATCC 25259, and *G. sulfurreducens* PCA; acidophiles, *C. burnetii* RSA 493 and *A. ferrooxidans* ATCC 23270. Also included in this group for comparison is the acid-tolerant *H. pylori* 26695.

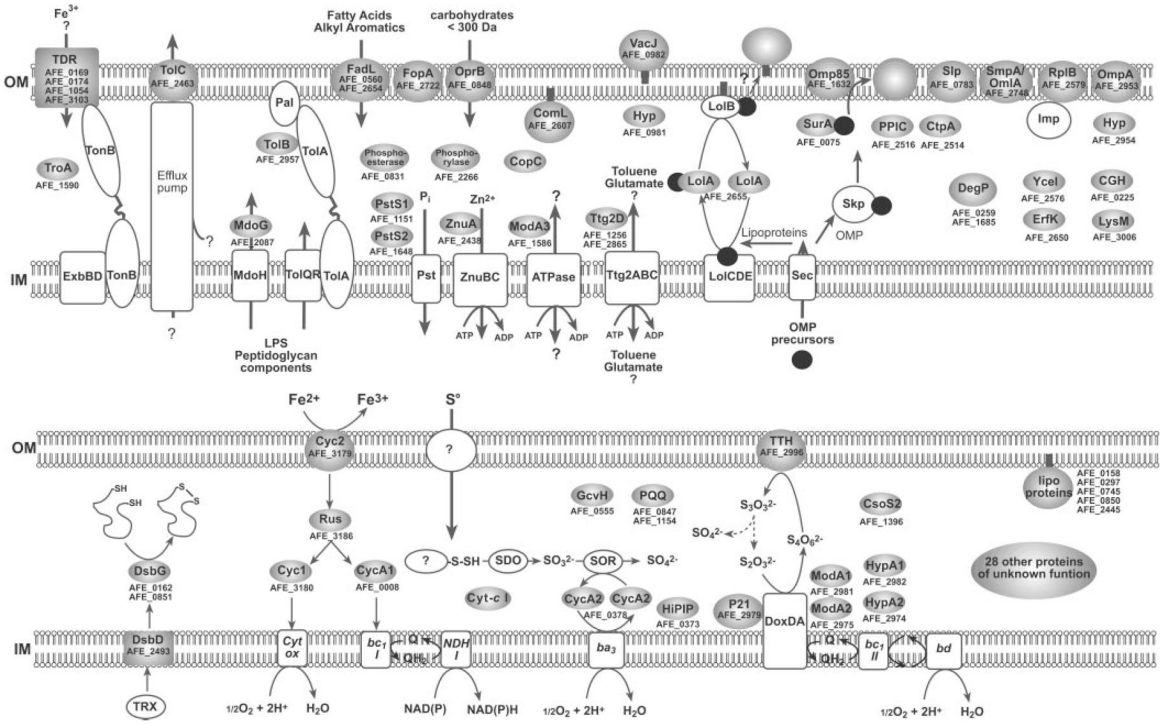

Fig. 4. Schematic location and suggested putative functions of the majority of the proteins identified in the periplasmic fraction from *A. ferrooxidans*

Most of the proposed localizations and possible roles of the indicated proteins are based on previously reported studies in *E. coli* and other microorganisms (1, 33, 40, 43, 73). The proteins shaded in *gray* were identified experimentally by proteomics analysis of the periplasmic fraction. Transport functions are highlighted in the *upper part*, and energy metabolism is in the lower portion of the figure. The OMPs are shown in their final destination into the outer membrane. *Black circles* represent OMP precursors in transit to the outer membrane. The order in which the different proteins are included in this schematic is arbitrary.

Table I

Proteins with predicted export signals identified in the periplasmic fraction of *A. ferrooxidans* ATCC 23270 grown in thiosulfate

AFE, predicted protein-coding genes from TIGR-Comprehensive Microbial Resource. Names of proteins correspond to those assigned by TIGR. Sec, SignalP prediction; Tat, TatP prediction; SecP, SecretomeP prediction. Molecular weight, pI, and export signal prediction was done with sequences from a local database.

AFE	Function/similarity	Molecular weight/pI	Export signal	No. unique peptides	Sequence coverage
					%
Transport and binding proteins					
1151	Phosphate ABC transporter, periplasmic phosphate- binding protein PstS2	38.4/9.3	Sec	16	47
1648	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS1	36.2/9.41	Sec	11	45
174	TonB-dependent receptor	86.9/9.19	Sec	6	10
1054	TonB-dependent receptor	85.3/6.53	Sec	20	37
1590	Iron compound ABC transporter, periplasmic iron-binding protein, putative	38.4/9.72	Sec	3	10
2438	Cation ABC transporter, periplasmic cation-binding protein, putative	33.5/8.85	Sec	6	38
2957	Tol-Pal system βpropeller repeat protein TolB	45.5/8.76	Sec	14	54
3103	TonB-dependent receptor	84.8/8.96	Sec	7	9
848	Carbohydrate-selective porin, OprB family	40.7/7.05	Sec	5	15
1256	Toluene tolerance protein, putative	25.2/9.08	Sec	2	9
2865	Toluene tolerance protein, putative	23.5/9.33	Sec	8	62
1586	ABC transporter, periplasmic substrate-binding protein, putative	37.3/9.18	Sec	13	53
2975	Periplasmic solute-binding protein, putative	36.6/8.73	Sec	17	73
2981	Periplasmic solute-binding protein, putative	36.7/9.1	Sec	21	59
Cell envelope					
2087	Periplasmic glucan biosynthesis protein, MdoG	57.4/7.38	Sec	2	7
2621	Pilin, putative	17.7/5.09	SecP	1	5
3018	Pilus chaperone protein, putative	30/11.54	Sec	5	27
158	Lipoprotein, putative	15.5/11.3	Sec, LipoP	2	20
297	Lipoprotein, putative	10.2/9.42	Sec, LipoP	3	49
560	Outer membrane protein, OMPP1/FadL/TodX family	47/8.59	Sec	2	9
745	Lipoprotein, putative	43.7/9.35	SecP	14	45

AFE	Function/similarity	Molecular weight/pI	Export signal	No. unique peptides	Sequence coverage
					%
783	Outer membrane lipoprotein Slp, putative	20.1/6.28	SecP, LipoP	2	18
850	Lipoprotein, putative	16.2/8.49	Sec, LipoP	6	47
982	VacJ lipoprotein	30.2/6.97	Sec, LipoP	1	3
1632	Outer membrane protein, OMP85 family	86.6/8.9	Sec	7	12
2445	Lipoprotein, putative	13.8/10.03	Sec, LipoP	5	41
2579	Rare lipoprotein B, putative	18.9/10.61	Sec LipoP	1	5
2654	Outer membrane protein transport protein, OMPP1/FadL/TodX family	36.9/5.92	Sec	8	28
2722	OmpA family protein	22.3/9.4	Sec	5	24
2748	Lipoprotein, SmpA-OmlA family	7.9/9.16	SecP	2	22
2953	Peptidoglycan-associated lipoprotein, putative	19.6/6.4	Sec, LipoP	2	13
2996	Outer membrane protein	55/8.93	Sec	24	55
Energy metabolism					
555	Glycine cleavage system H protein, putative	15.2/4.74	SecP	3	22
8	Cytochrome c_4 (CycA-1)	27.5/9.65	Sec	1	3
373	Iron oxidase (Iro)	10.8/9	Tat	3	41
378	Cytochrome c_4 (CycA-2)	26.2/9.95	Sec	4	13
3179	Cytochrome *c* (Cyc2)	52.8/5.4	Sec	5	15
3180	Cytochrome c_{552} (Cyc1)	25.2/9.2	Sec	6	37
3186	Rusticyanin (Rus)	19.9/8.85	Sec	15	94
Protein fate					
2463	Type I secretion outer membrane protein, TolC family	50.4/6.4	Sec	1	2
259	Serine protease, DO-DeqQ family	53.4/7.3	Sec	8	21
1685	Serine protease, DO-DeqQ family	49.7/7.14	Sec	3	9
2514	C-terminal peptidase (CtpA)	50.8/8.93	Sec	2	5
75	Survival protein SurA (SurA)	52.3/6.42	Sec	11	29
162	Thiol:disulfide interchange protein DsbG, putative	31.8/8.54	Sec	3	16
2516	Peptidyl-prolyl cis-trans isomerase, PPIC-type	28/9.24	Sec	6	25
Cellular processes					
1399	Carboxysome peptide B	92.4/8.21	SecP	1	10
2607	Competence lipoprotein ComL, putative	30.2/8.93	Sec, LipoP	7	21

AFE	Function/similarity	Molecular weight/pI	Export signal	No. unique peptides	Sequence coverage
					%
Central intermediary metabolism					
2979	Sulfur-pyrite-thiosulfate-sulfide-induced protein (P21)	23.8/9.36	Sec	5	32
225	Choloylglycine hydrolase, putative	39.3/7.15	Sec	4	14
Protein synthesis					
491	Ribosomal protein L35	75/12.31	SecP	1	22
2701	Ribosomal protein S19	10.3/11.28	SecP	1	9
2717	Ribosomal protein L11	14.9/9.84	SecP	1	8
Unknown function					
831	Phosphoesterase family protein	60/6.26	Tat	14	33
2266	Phosphorylase, putative	39.5/7.28	SecP	4	13
169	TonB-dependent receptor domain protein	28.9/8.41	SecP	2	15
847	PQQ enzyme repeat domain protein	43.2/9.61	Sec	19	48
851	DsbG domain protein	22.3/8.42	Sec	3	19
1154	PQQ enzyme repeat domain protein	41.7/7.82	Sec	4	14
1396	Tat pathway signal sequence domain protein (carboxysome structural protein CsoS2)	78.7/9.32	Tat	3	5
2576	YceI-like family protein	25.2/9.28	Sec	10	42
2650	ErfK-YbiS-YcfS-YnhG family protein	37.6/7.77	Sec	1	3
2753	Thiol:disulfide interchange domain protein	21.9/6.96	Sec	5	30
2974	Tat pathway signal sequence domain protein	29.5/6.24	Tat	7	44
2982	Tat pathway signal sequence domain protein	29.7/6.35	Tat	7	47
3006	LysM domain protein	37.6/9.62	Sec	7	22

Table II

New periplasmic conserved proteins, unique periplasmic proteins, and periplasmic proteins not previously annotated in the genome of A. ferrooxidans ATCC 23270

Conditions and sequence analysis were the same as in Table I.

AFE	Function/similarity	Score	E value	Molecular weight/pI	Export signal	No. unique peptides	Sequence coverage
							%
Hypothetical proteins							
136	Conserved hypothetical protein			63.2/9.78	Sec	4	8
163	Conserved hypothetical protein			59.6/9.4	Sec	3	7
278	Conserved hypothetical protein			23.9/9.04	Sec	1	4
981	Conserved hypothetical protein			24.2/8.92	Sec	6	29
2570	Conserved hypothetical protein			27.4/9.98	SecP	2	11
2954	Conserved hypothetical protein			28.9/8.91	Sec	3	14
3181	Conserved hypothetical protein			19.9/8.54	Sec	6	42
1060	Conserved domain protein			39.3/7.29	Sec	12	47
1061	Conserved domain protein			10.8/5.66	Sec	7	63
2507	Conserved domain protein			28/8.04	SecP	1	4
2910	Conserved domain protein			52.2/9.31	Sec	2	6
157	Hypothetical protein			31.3/10.79	Sec	3	11
292	Hypothetical protein			15.5/9.3	Sec	8	54
457	Hypothetical protein			15.4/9.74	Sec	6	52
610	Hypothetical protein			10.9/12.24	Sec	3	31
826	Hypothetical protein			17.8/9.72	Sec	3	25
1110	Hypothetical protein			16.1/10	Sec	4	20
2111	Hypothetical protein			21.4/9.94	Sec	4	25
2167	Hypothetical protein			25.4/9.88	SecP	3	15
2418	Hypothetical protein			27.9/9.55	Sec	1	4
2429	Hypothetical protein			46.3/9.04	Sec	7	21
2434	Hypothetical protein			44.5/8.78	Sec	2	7
2770	Hypothetical protein			19.9/5.04	Sec	2	14
2928	Hypothetical protein			14.8/7.8	Sec	3	32

AFE	Function/similarity	Score	E value	Molecular weight/pI	Export signal	No. unique peptides	Sequence coverage
							%
2994	Hypothetical protein			49.8/9.1	Sec	13	44
3060	Hypothetical protein			20.3/10.14	Sec	3	25
3099	Hypothetical protein			21.3/8.56	Sec	3	18
3197	Hypothetical protein			14.5/9.48	Sec	8	50
ORFs not annotated by TIGR							
	Copper resistance protein CopC (Burkholderia sp. 383)	85.9	4*e*–16	13.5/10.17	Sec	4	45
	Putative sterol carrier protein (*T. denitrificans* ATCC 25259)	120	3*e*–26	20.5/9.4	Sec	1	10
	Cytochrome *c*, class I (*Anaeromyxobacter dehalogenans* 2CP-C)	74.3	5*e*–12	32.2/9.99	SecP	12	31
	Hypothetical protein			15.4/7.25	Sec	3	33
	Hypothetical protein			10.4/9.43	Sec	1	11
	Hypothetical protein			15.3/10.28	Sec	4	30
	Hypothetical protein			16.8/9.33	Sec	9	69