A novel gene encoding a sulfur-regulated outer membrane protein in *Thiobacillus ferrooxidans*

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Abstract

Thiobacillus ferrooxidans is a Gram-negative chemolithotrophic bacterium able to oxidize ferrous iron, elemental sulfur and inorganic sulfur compounds. The oxidation of sulfur by *T. ferrooxidans* resulted in an expression of some outer membrane proteins (OMPs) at a level higher than that observed during ferrous iron oxidation. Among these OMPs, a protein with a molecular mass of 54 kDa was purified and 18 amino acids of the N-terminal sequence determined. Using a 54 bp PCR generated DNA product as a probe for the protein, we isolated a 4.5 kb *Pst* I DNA chromosomal fragment containing the corresponding gene. Sequencing 2169 bp of this fragment revealed the open reading frame codifying for the protein, consisting of 467 amino acids and a molecular mass of 49 674 Da. The mature protein was produced by the removal of a 32 amino acid signal peptide-like sequence from the N-terminus of a 499 amino acid peptide. Although no significant homology with any known protein has been found and its physiological role remains unclear, its high expression on sulfur substrates suggests a role in sulfide mineral oxidation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Thiobacillus ferrooxidans; Sulfur oxidation; Outer membrane proteins

1. Introduction

Thiobacillus ferrooxidans is a Gram-negative, acidophilic, chemolithotrophic bacterium able to

derive energy for growth from the oxidation of ferrous to ferric iron and elemental sulfur or reduced inorganic sulfur compounds to sulfate using oxygen as electron acceptor. This peculiar metabolism makes it one of the most important microorganisms involved in bioleaching, i.e. the biological solubilization of metal ions from

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sulfidic ores (Brierley, 1978). This process was generally believed to occur both by a direct biological, and an indirect chemical, mechanism (Lundgren and Silver, 1980) even though the contribution of the direct mechanism, involving bacterial catalysis in the leaching of sulfide minerals, has been recently refuted (Sand et al., 1995; Fowler and Crundwell, 1998).

Many studies have been done on the mechanism by which this lithoautotrophic bacterium obtains its energy. With regard to Fe^{2+} utilization, which has been extensively investigated, several oxido-reductase proteins involved in its oxidation have been identified and functionally characterized (Yamanaka and Fukumori (1995) and references therein). Conversely, no clear cut pathway has emerged for the biological oxidation of elemental sulfur and inorganic sulfur compounds even though it has been investigated by several authors (Suzuki and Werkman, 1959; Bacon and Ingledew, 1989; Sugio et al., 1991; Lorbach et al., 1992; Kuenen et al., 1993; Kelly et al., 1997). However, it is widely accepted that in elemental sulfur utilization the adhesion of bacteria to insoluble sulfur particles or a sulfidic mineral surface is required for the most efficient oxidation. The adhesion process of *T. ferrooxidans* to the mineral depends on the biochemical properties of the microorganism and the chemical–physical features of the substrate. In this regard, surface hydrophobicity (Devasia et al., 1993) and extracellular polymeric substances (Gehrke et al., 1998) were found to affect the adhesion of *T. ferrooxidans* to sulfur-containing minerals. Membrane components such as outer membrane proteins could also play a role both in adhesion and in elemental sulfur oxidation (Rodríguez et al., 1986). We have previously observed that sulfur grown cells exhibited an increased expression of certain outer membrane proteins (Buonfiglio et al., 1993) and, more recently, a protein localized on the flagella of *T. ferrooxidans* was demonstrated to be involved in the binding of the microorganism to elemental sulfur (Ohmura et al., 1996).

In this paper we report the purification of an outer membrane protein up-regulated as a result of growth in the presence of sulfur substrates, and the sequencing of its corresponding gene.

2. Materials and methods

2.1. Bacterial strains

T. ferrooxidans strains MSR (Valenti et al., 1990) and ATCC 23270 were used in this study. *Escherichia coli* DH5α strain (Sambrook et al., 1989) was used for genetic manipulations and grown in Luria–Bertani (L.B.) medium (Bertani, 1951) at 37°C. Recombinant *E. coli* DH5α clones, derived from transformation experiments, were cultured in L.B. medium supplemented with ampicillin.

2.2. Growth and oxidation of T. ferrooxidans on different substrates

T. ferrooxidans strains were grown in 9K liquid medium (Silverman and Lundgren, 1959) at pH 2.5 supplemented with either 44.2 g l^{-1} $FeSO_4 \cdot 7H_2O$, 20 g l^{-1} S°, or 10 g l^{-1} pyrite, or in 9K liquid medium at pH 4.5 supplemented with 10 g l^{-1} $Na_2S_2O_3 \cdot 5H_2O$. Cultures were incubated at 30°C under aerobic conditions. Growth was measured by the determination of proteins as estimated according to Lowry et al. (1951) using bovine serum albumin as a standard. Briefly, samples of culture taken at different times, were filtered through a Whatman No. 1 filter paper to remove insoluble materials, and the bacteria collected from the filtrate by centrifugation were washed three times in 1 mM H_2SO_4. The pelleted bacteria were suspended in 1 N NaOH, hydrolized for 1 min at 100°C and assayed. Iron oxidation was determined by volumetrically titrating ferrous iron present in the culture medium with 0.1 N $KMnO_4$ at 60°C in H_2SO_4 solution. A Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) in a 1.6 ml water-jacketed chamber maintained at 30°C was used to monitor inorganic sulfur compound oxidation according to the experimental procedure described by Lorbach et al. (1992).

2.3. Extraction and electrophoresis of outer membrane proteins (OMPs)

To obtain purified OMPs, the cells were disrupted by sonication (Buonfiglio et al., 1993) and the membranes separated on sucrose gradients (Mizushima and Yamada, 1975). Outer membrane proteins were resolved by polyacrylamide–sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein concentration was estimated according to Bradford (1976).

2.4. Protein purification and N-terminal sequencing

After electrophoresis, the proteins were directly electroblotted onto a sheet of methanol rinsed polyvinylidene difluoride (PVDF) membrane (Immobilon™-P, Millipore) according to the procedure described by Matsudaira (1987). The N-terminal amino acid sequence was determined by the automated Edman degradation method using a Perkin-Elmer Applied Biosystem 476A sequencer.

2.5. DNA extraction and electrophoresis

T. ferrooxidans genomic DNA was extracted according to the protocol of Silhavy et al. (1984). Plasmid DNA extraction from *E. coli* DH5α recombinant clones was performed according to the procedure of Birnboim and Doly (1979).

2.6. Generation of a DNA probe by PCR

Two sets of 17-mer degenerate oligonucleotides were designed based on N-terminal sequencing and used as primers in a PCR reaction with genomic *T. ferrooxidans* MSR DNA as the template (Compton, 1990). One set of degenerate oligonucleotides was made to the sense strand of the DNA (5′ GTG(C,A,T)GCG(C,A,T)GTG-(C,A,T)CCG(C,A,T)ATGGA 3′ 256-fold degenerate) while the other set was made to the anti-sense strand and in the opposite direction (5′ GGG(A)TGG(C,A,T)AC(T)G(C,A,T)ACG(C,A,T)GTG(C,A,T)CG 3′ 1024-fold degenerate).

The cycling parameters for the Perkin-Elmer 480 DNA thermal cycler were as follows: denaturing, 96°C, 1 min; annealing, 37°C, 1 min; primer extension, 72°C, 2 min 30 s for two cycles followed by 35 cycles of 94°C, 1 min; 50°C, 30 s; and 72°C, 1 min. The PCR reaction was analysed by SDS-PAGE.

2.7. Cloning and analysis of the PCR product

Basic molecular biology techniques were used for the cloning and sequencing experiments (Sambrook et al., 1989). The PCR product (approximately 54 bp) was purified and ligated to *Sma*I-digested pT7/T3α18 (Boehringer Mannheim). The ligation mixture was used to transform competent (via CaCl$_2$) *E. coli* DH5α. Transformants were scored on L.B. plates containing 100 μg ml^{-1} ampicillin, 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG), and 0.004% 5-bromo-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Positive clones were further identified by double digestion of the recombinant plasmid DNA with *Eco*RI and *Hin*dIII restriction enzymes.

2.8. Digoxigenin probe labeling and Southern hybridization

The DNA fragment ligated to pT7/T3α18 was labeled with digoxigenin by PCR adding to the reaction DIG-11-dUTP during the amplification process using the DIG DNA labeling and detection kit (Boehringer Mannheim). *T. ferrooxidans* genomic DNA was digested with different restriction enzymes, subjected to gel electrophoresis and transferred onto Hybond-N nylon membrane (Amersham) by Southern blotting. Membrane hybridization, washing and detection were performed according to the manufacturer protocol using the chemiluminescent substrate Lumigen™ PPD.

2.9. Colony hybridization

Colony hybridization experiments were carried out after transferring recombinant *E. coli* DH5α clones from master plates to Hybond-N nylon

membranes. The colonies were lysed and DNA was fixed to the filters by baking at 80°C for 2 h (Perbal, 1988). Hybridization was performed according to Perbal (1988) using the PCR product radiolabeled with $[\gamma\text{-}^{32}P]ATP$ as the probe.

2.10. Nucleotide sequence determination and analysis

The double strand sequence was determined using an ABI Taq dyedeoxy cycle sequencing kit in conjuction with an ABI 373 A DNA sequencer. Sequences were analyzed using Gene-Works, GeneJockey, MacVector, and AsasemblyLIGN. Accession number AF005208.

3. Results

3.1. Growth and oxidation of sulfur substrates by T. ferrooxidans

A series of experiments has been carried out by culturing *T. ferrooxidans* on media containing different energy sources. At the first passage the growth of *T. ferrooxidans* MSR, routinely cultured on ferrous iron, was delayed on sulfur compounds, while after the third passage bacteria reached a maximal production of biomass (100, 88 and 52 μg ml^{-1} on elemental sulfur, thiosulfate and pyrite, respectively, after 144 h in comparison with 22 μg ml^{-1} after 84 h on Fe^{2+} ions) and a maximal oxidation rate (V_{max}) measured by O_2 consumption. This V_{max} was correlated with the sulfur substrate utilized and varied from 320 U for elemental sulfur, 112 U for thiosulfate to 75 U for pyrite. These data clearly indicated that the lag phase observed during the first shift of bacteria from ferrous iron to sulfur compounds was required for the synthesis of new molecules, among which could very well be OMPs, essential for sulfur utilization.

3.2. OMP analysis

The outer membrane protein profile of *T. ferrooxidans* MSR subcultured for three serial passages on ferrous iron and elemental sulfur is
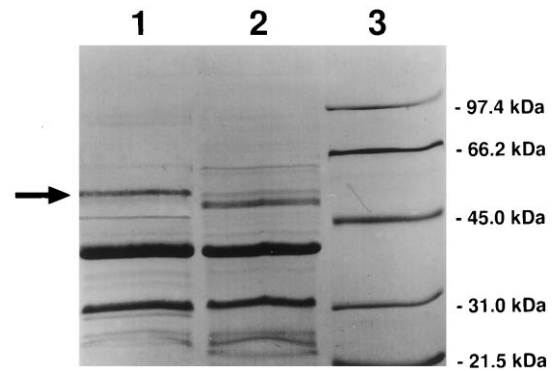

Fig. 1. Coomassie blue stained SDS-PAGE of *T. ferrooxidans* MSR grown on S° (lane 1) and Fe^{2+} (lane 2). Lane 3, molecular weight standards. The arrow indicates the 54 kDa protein.

shown in Fig. 1. As expected from the data reported above, the shift of *T. ferrooxidans* MSR from Fe^{2+} to S° liquid medium modulates the expression of some OMPs. In particular, an increased synthesis of an OMP with a molecular mass of 54 kDa was observed only when bacteria were adapted to grow on elemental sulfur (at the third passage). The presence of a corresponding band in the protein profile of *T. ferrooxidans* MSR grown on ferrous iron suggests that the 54 kDa protein is also expressed with this energy substrate, but at a lower level similar to that observed for *T. ferrooxidans* MSR at the first passage on elemental sulfur. The same protein also underwent an increased expression when the microorganism was adapted to grow on thiosulfate or pyrite as the sole energy source, although
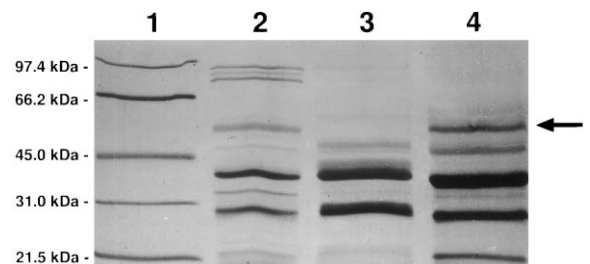

Fig. 2. Coomassie blue stained SDS-PAGE of OMPs of *T. ferrooxidans* MSR grown on pyrite (lane 2), Fe^{2+} (lane 3) and sodium thiosulfate (lane 4). Lane 1, molecular weight standards. The arrow indicates the 54 kDa protein.

to a different extent (Fig. 2). Similar results were also obtained for *T. ferrooxidans* ATCC 23270 (data not shown). Elemental sulfur production probably deriving either from biological oxidation or chemical dismutation was also observed to occur during microbial growth on thiosulfate.

3.3. Identification and cloning of the gene for the OMP

The N-terminal amino acid sequence of the outer membrane protein was determined to be: Ala–Val–Ala–Val–Pro–Met–Asp–Ser–Thr–Gly–Pro–Tyr–Arg–Thr–Val–Ser–His–Pro. To identify the gene coding for the 54 kDa protein, two sets of 17-mer degenerate oligonucleotides were synthesized on the basis of the amino acid N-terminal sequence and used as PCR primers. Polyacrylamide gel electrophoresis of the PCR reaction revealed a major product of about 54 bp. Hybridization of this DNA fragment with genomic DNA of *T. ferrooxidans* strains MSR and ATCC 23270 digested with *Bam*HI, *Hin*dIII, *Pst*I and *Pvu*I allowed the localization of the gene on a *Pst*I DNA restriction fragment of approximately 4.5 kb. The results obtained also demonstrated that the gene is located on the bacterial chromosome. In fact, *T. ferrooxidans* MSR strain harbours a unique 20 kb plasmid (pTFO) possessing a single *Pst*I site (Valenti et al., 1990).

T. ferrooxidans MSR DNA fragments obtained by digestion with *Pst*I ranging from 4 to 5 kb were purified and ligated to pT7/T3α18. The ligation mixture was used to transform *E. coli* DH5α and the resulting transformants were recovered on L.B. plates containing 100 μg ml^{-1} ampicillin, 0.5 mM IPTG, and 0.004% X-Gal. Hybridizing the colony lifts deriving from the master plates with the [γ-^{32}P]ATP labeled 54 bp probe resulted in the isolation of five positive clones, termed P1–P5. A restriction enzyme analysis confirmed that all the five positive clones harbored a plasmid containing a DNA insert of the predicted size, 4.5 kb.

3.4. DNA sequence and analysis

Sequencing 2169 bp of the 4.5 kb chromosomal fragment revealed the open reading frame coding for the 54 kDa protein (Fig. 3). Three possible start codons are present which begin at nucleotides 469, 496 and 514. The most likely ORF encoding a 499 amino acid protein with a deduced molecular mass of 53 089 Da begins with an ATG at position 514 and extends 1497 nucleotides to a TAG stop codon. This ORF is preceded by an excellent prokaryotic ribosome binding site (5′-GGAGA-3′) (Shine and Dalgarno, 1975) located 6 bp upstream of the ATG codon. The −10 sequence of a putative promoter is 104 nucleotides upstream of the start codon. Downstream the TAG stop codon, inspection of the noncoding region revealed an inverted repeat sequence with three unbounded bases (Fig. 3, double underlined) that likely serves as a transcriptional terminator.

The N-terminal sequence of the purified mature protein agreed with the peptide sequence deduced from its cloned gene, but this sequence begins 96 nucleotides downstream from the proposed start codon. The 32 amino acid N-terminal polypeptide, whose sequence resembles a typical signal peptide, must be post-translationally removed from the protein. This sequence is formed by a positively charged N-terminal end (Arg and Lys), a central hydrophobic core sequence rich in leucine residues and a conserved signal peptide cleavage site, formed by amino acids with small side chains (Ala and Ser) and preceded by a proline residue. All these characteristics are consistent with those proposed for prokaryotic signal peptides mediating the transmembrane passage of proteins (von Heijne, 1994).

After the removal of the 32 amino acid signal polypeptide the deduced molecular mass of the 467 amino acid mature protein is about 50 kDa (49 674 Da). The presence of only one cysteine residue suggests that no disulfide bridge is present in the protein.

A Kyte and Doolittle (1982) hydropathy plot of the deduced amino acid sequence of the complete gene is shown in Fig. 4. The N-terminal region of the protein exhibited a profile typical of signal peptides. Other significant features of the protein were not apparent.

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TGG TAT CAG CCG TAA GCA ACT GCA CAC CCG CAT GAA AAA TCT CGG ACT GAT CGT CGA CCG TGA AGA TGT GCC GGG    75

CGG CGA GAA GCT ATA ATT TCG AGC GAG GCG CCA GTC TGG CCC CTT ACT TGG CAA GGC CAA ATG AGA TCG AAA TAC   150

GCG CTC GTT GGC CCT TTT GGG ACA ACT TCA CGG CCG ACC TGT GCC GTT TGG CCC TTC CCC ACT CCT TCC AGA TAA   225

CCC ACG TTG TAA GAA TAA CAA AAT AAA ACA GCA TCC TGT AAT CAT TAT GCG GCT GGC ACA ATA CAT GCA TAA AAC   300

ATC TCG TGC TGC CGC GGG ATT CTC ATT TTC ATT TAC TCA TTG AAT ATC ATA AGC GGG ATG CAG CCA TAA ATA TAA   375
                    -35               -10
CGC TGG TTG TGT TAT TGG GAG CGA GCA TTT AAT TCT GTT GAT ATC GTT TAT GGG AAT AAC AAA AGT GGA GAC GCA   450
                                                                            �\_\_\_▶ OMP gene
GCT GAA AAA GCG AAG CTG ATG ACA GAG TAC GTT ATA CCT CAC CAA ATG GAT TGG AGA CTT GAT ATG CCA AGT ATT   525
                                                                            Met Pro Ser Ile

GTA CGT AAC CAT GGT CCA CAT AAC AAA ATT CTT TTA TCG GCA TTA TTG CTG GCC CTC TTC GGC TGG GTG CCG TTG   600
Val Arg Asn His Gly Pro His Asn Lys Ile Leu Leu Ser Ala Leu Leu Leu Ala Leu Phe Gly Trp Val Pro Leu

GCG TCC GCC GCT GTT GCT GTT CCA ATG GAT TCA ACC GGG CCC TAT CGA ACG TTA TCC CAT CCG GAA AAC GCA CCA   675
Ala Ser Ala Ala Val Ala Val Pro Met Asp Ser Thr Gly Pro Tyr Arg Thr Val Ser His Pro Glu Asn Ala Pro

TCT GGC GTG GAT GCC GGT GTC GGC CCA TCC GAA TGG ACA CAC GCA TAC GCC AAT CCC GCC CAC AAT GCT GCA TTT   750
Ser Gly Val Asp Ala Gly Val Gly Pro Ser Glu Trp Thr His Ala Tyr Ala Asn Pro Ala His Asn Ala Ala Phe

CCG GTC CCT GAC GAC GCG CCG GAA TGG ATA AGG AAC GGC GTA TCA TGG TTG TTT CCG GAA GCC CGT GCA TGG CCT   825
Pro Val Pro Asp Asp Ala Pro Glu Trp Ile Arg Asn Gly Val Ser Trp Leu Phe Pro Glu Ala Arg Ala Trp Pro

CTG GCA AAC CCG CCG TTC GGG AGT AAG ACC TAT GGG GCC GCA GAA GCT TCC GTA ACA CAA ACC CAG TTC TAT GGA   900
Leu Ala Asn Pro Pro Phe Gly Ser Lys Thr Tyr Gly Ala Ala Glu Ala Ser Val Thr Gln Thr Gln Phe Tyr Gly

AAC GCA CTG GGG CCA TCG GTG GTC GAC GGT GTG GTC TAT GCG GAA AGC GAT GAT ATG TTT GCA TAT GCG GTT AAC   975
Asn Ala Leu Gly Pro Ser Val Val Asp Gly Val Val Tyr Ala Glu Ser Asp Asp Met Phe Ala Tyr Ala Val Asn

GCA AAA ACC GGG AAA CTC ATC TGG CGC GCG AGT CCT GTC GGA AAT AAT TTG ATG GGC AAC CCG TTG GTC ATT GGA  1050
Ala Lys Thr Gly Lys Leu Ile Trp Arg Ala Ser Pro Val Gly Asn Asn Leu Met Gly Asn Pro Leu Val Ile Gly

AAT ACA GTA TAT CTA TCC GCC GGC AGT GTA GCC TTC AAC TTC GCC AAC GTC TTG CGA TAT GCC CAC AAT CCA TCG  1125
Asn Thr Val Tyr Leu Ser Ala Gly Ser Val Ala Phe Asn Phe Ala Asn Val Leu Arg Tyr Ala His Asn Pro Ser

GCC TCA GCG CGA GGC CTG AAT GTG AGT TTC AAC GGC ATT TAT GCC CTT AAC CGC AGC AAC GGC AAG CTT CTT TGG  1200
Ala Ser Ala Arg Gly Leu Asn Val Ser Phe Asn Gly Ile Tyr Ala Leu Asn Arg Ser Asn Gly Lys Leu Leu Trp

TAC TTC GCG ACG CCT GGC GAA ACC ATG GCG ACA CCG GCC TAT GAC AAT AAC ACC TTG TTT ATT GCC GAT GGC GCG  1275
Tyr Phe Ala Thr Pro Gly Glu Thr Met Ala Thr Pro Ala Tyr Asp Asn Asn Thr Leu Phe Ile Ala Asp Gly Ala

GGA AAT GCG TTT GGG ATT AAT GCC ACG ACT GGC AAG CAG GTC TGG AAA ACC CAT GTG GGG GGC ATG GAC AAC ATG  1350
Gly Asn Ala Phe Gly Ile Asn Ala Thr Thr Gly Lys Gln Val Trp Lys Thr His Val Gly Gly Met Asp Asn Met

TCC AGT GTG ACA GCG TAT CGG CAC AAT ATC TAT TTT GCT ATG GCT ATC AAG CCA TAT CTT TAT TGT CTG AAT GAA  1425
Ser Ser Val Thr Ala Tyr Arg His Asn Ile Tyr Phe Ala Met Ala Ile Lys Pro Tyr Leu Tyr Cys Leu Asn Glu

TCC AAC GGG CAT ATC GTA TGG AAA GGG ACG ATA CCG GGT GCC AGT AAT ACT GGT ATT GGC GAC GTA TCT CCG GCG  1500
Ser Asn Gly His Ile Val Trp Lys Gly Thr Ile Pro Gly Ala Ser Asn Thr Gly Ile Gly Asp Val Ser Pro Ala

GCG GCC GAT GGT GTT GTC GTA CTC GAT GCG ACA ACG AAA CCG CAA GCC AAT AAA AAG GCC ATG TTC AGC AAC GTC  1575
Ala Ala Asp Gly Val Val Val Leu Asp Ala Thr Thr Lys Pro Gln Ala Asn Lys Lys Ala Met Phe Ser Asn Val

ATT CGC GCA TTT GAT GCC AAG ACC GGC GCG GTT CTG TGG ACC AGA AAC ATG GGT AGC GGA GGA AAA ATA CCC GCG  1650
Ile Arg Ala Phe Asp Ala Lys Thr Gly Ala Val Leu Trp Thr Arg Asn Met Gly Ser Gly Gly Lys Ile Pro Ala

TTT AAA GGG GGC GTT CCC ATG ATC CAC AAC AAC ATC GTA TAT GTG GGC AAT CCC GTC GCG TCA ACA TAC CAG GCG  1725
Phe Lys Gly Gly Val Pro Met Ile His Asn Asn Ile Val Tyr Val Gly Asn Pro Val Ala Ser Thr Tyr Gln Ala

TAT GAA CTT AAA ACG GGC AAG CTG CTC TGG ACA TGG CAT GTC CCA ACC AAA GTT GCA GCA GGC GCG GGG CGT TCG  1800
Tyr Glu Leu Lys Thr Gly Lys Leu Leu Trp Thr Trp His Val Pro Thr Lys Val Ala Ala Gly Ala Gly Arg Ser

GCT CCA ACC TAT TAC AAA GGA TTG CTG TAC ATA ACT ACC GGG CAG TAC ATA TAT GTT GTG AAT CCC GCC ACC GGA  1875
Ala Pro Thr Tyr Tyr Lys Gly Leu Leu Tyr Ile Thr Thr Gly Gln Tyr Ile Tyr Val Val Asn Pro Ala Thr Gly

AAG GAA CTT CAC CAA CAT CAT ATC GGT GGT CAG TTC GGG ATC GAA AGC CCC GTA ATA GTG GGG GGC ACC GTC TAC  1950
Lys Glu Leu His Gln His His Ile Gly Gly Gln Phe Gly Ile Glu Ser Pro Val Ile Val Gly Gly Thr Val Tyr

CTG ACC AAC TCC TGG GAC TGG ATC ATG GCG ATT CCG CTA AAA ACG ATA AGC ATG GCA GTT AGG TTT TCA CAA CGT  2025
Leu Thr Asn Ser Trp Asp Trp Ile Met Ala Ile Pro Leu Lys Thr Ile Ser His Gly Ser   *

GTT ATC GGC CGG ACC GAT AAC ACG TAA CCA GCC AGC CAT CTG CCT CGT TAG CTT TCT GGT CAA CGA AAC CAG CAC  2100

GCC CGG GGC GAT GTC TGA AGG AGA TAG ATA TGG GGA AGA GGA AAC AAC CAA AAA AAT ATG CAA TAT ATG          2169
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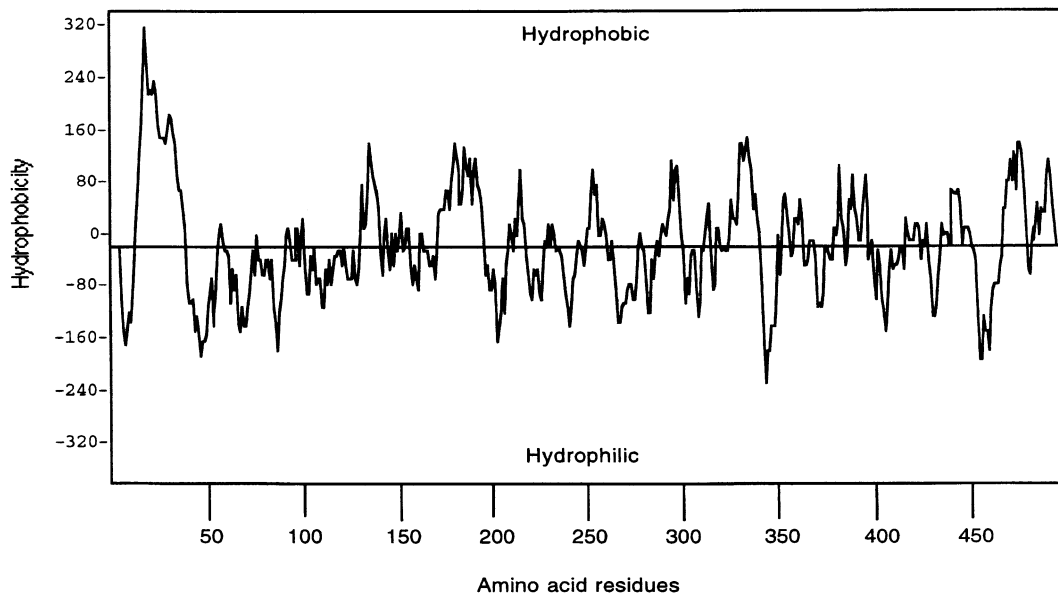
<p align="center">Fig. 3.</p>

Fig. 4. Hydropathy profile of the 53 089 Da OMP (includes the signal peptide) prepared using the deduced amino acid sequence. Hydrophobic values were calculated by the method of Kyte and Doolittle (1982).

3.5. Gene/protein analysis

Computer searches of available data bases revealed that the gene coding for the 50 kDa OMP has no significant homology with any other known prokaryotic genes/proteins.

4. Discussion

The oxidation of elemental sulfur or sulfide minerals by *T. ferrooxidans* requires the direct interaction of the bacteria with the surface of the substrate particles. Due to the physico–chemical nature of an insoluble growth substrate such as sulfur, the external surface of the bacterium undergoes several changes, which render the microorganism capable of adhering to the insoluble particles (DiSpirito et al., 1983).

As previously described (Rodríguez et al., 1986; Buonfiglio et al., 1993; Osorio et al., 1993) the shift of *T. ferrooxidans* from a liquid medium containing Fe^{2+} as the energy substrate to one with S° modulates the expression of some external proteins of molecular mass ranging from 45 to about 55 kDa. This study demonstrates the increased expression of a specific *T. ferrooxidans* 50 kDa protein when the bacterium is grown on elemental sulfur or inorganic sulfur compounds in comparison with the growth on ferrous iron. The increased expression was evident not only with S° but also with either thiosulfate or pyrite. Since elemental sulfur production occurred during growth on thiosulfate, it is possible to hypothesize that the 50 kDa protein is overexpressed in the presence of S°. On the other hand, it is also likely that the protein is up-regulated in response to

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Fig. 3. Nucleotide and deduced amino acid sequence of the *T. ferrooxidans* OMP gene. The arrow indicates the direction and initiation of the translation. The − 10 and − 35 consensus sequences of the putative promoter are reported in bold type. An asterisk indicates the stop codon. The putative ribosome binding site is underlined. The boxed region indicates a signal peptide-like sequence. The inverted repeat sequence acting as a transcription terminator is double underlined.

different sulfur containing compounds. The increased expression of the 50 kDa protein was more evident after three passages of *T. ferrooxidans* MSR, usually cultured on ferrous iron, on sulfur containing media. The results obtained for *T. ferrooxidans* MSR were also confirmed for *T. ferrooxidans* ATCC 23270, the type strain, indicating that the characteristic is potentially widespread among different strains of the organism. Thus, it seems likely that the 50 kDa OMP could play an important role in the sulfur utilization by this microorganism, likely acting in the preliminary step which requires the attachment of bacteria to insoluble sulfur particles.

This research reports, for the first time, the sequencing of the gene coding for the above protein. All of the elements necessary for the expression of the protein are evident in the DNA sequence, i.e. a promoter, a ribosome binding site, and a transcriptional terminator signal. In addition, a signal peptide-like sequence was identified at the N-terminal region of the protein. All of the characteristics of this sequence are consistent with those proposed for prokaryotic signal peptides mediating transmembrane passage of proteins. A hydropathy plot exhibited a profile typical of signal peptides for the N-terminal region. The leader is obviously removed by a proteolytic cleavage during or after the export of the protein to the surface of the cell.

In a recent study Ohmura et al. (1996) have identified a sulfur binding surface protein of 40 kDa in *T. ferrooxidans* ATCC 23270 grown on elemental sulfur. This surface protein, located on the flagella, has thiol groups and adheres to elemental sulfur powder. These authors detected in the same strain two other sulfur regulated surface proteins of 46 and 50 kDa.

The physiological role of the 50 kDa protein is unclear. At the moment, we do not know if the 50 kDa protein is involved in the substrate-dependent modification of the bacterial surface or if it might play a role in the binding and/or processing of sulfur particles. In addition proteins of defined function and with a similar sequence have not yet been reported.

Experiments are in progress in order to try to assign a function to this novel protein and to determine if the 50 kDa protein is present in other *Thiobacillus* spp.

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