The HiPIP from the acidophilic Acidithiobacillus ferrooxidans is correctly processed and translocated in Escherichia coli, in spite of the periplasm pH difference between these two micro-organisms

Patrice Bruscella,1 Laure Cassagnaud,1 Jeanine Ratouchniak,1 Gaël Brasseur,2 Elisabeth Lojou,2 Ricardo Amils3 and Violaine Bonnefoy1

1,2Laboratoire de Chimie Bactérienne1 and Laboratoire de Bioénergétique et Ingénierie des Protéines2, IBSM, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France
3Universidad Autonoma de Madrid, Centro de Biologia Molecular, Cantoblanco, Madrid, Spain

INTRODUCTION

One of the most studied bacteria that thrives in acidic mine drainage is Acidithiobacillus ferrooxidans, formerly Thiothrix ferrooxidans (Kelly & Wood, 2000; Leduc & Ferroni, 1994). It is characterized as a Gram-negative, acidophilic chemolithoautotrophic Acidithiobacillus ferrooxidans ATCC 33020 has been cloned and sequenced. This potential HiPIP was overproduced in the periplasm of the neutrophile and heterotroph Escherichia coli. As shown by optical and EPR spectra and by electrochemical studies, the recombinant protein has all the biochemical properties of a HiPIP, indicating that the iron–sulfur cluster was correctly inserted. Translocation of this protein in the periplasm of E. coli was not detected in a ΔtatC mutant, indicating that it is dependent on the Tat system.

The genetic organization of the iro locus in strains ATCC 23270 and ATCC 33020 is different from that found in strains Fe-1 and BRGM. Indeed, in A. ferrooxidans ATCC 33020 and ATCC 23270 (the type strain), iro was not located downstream from purA but was instead downstream from petC2, encoding cytochrome c1 from the second A. ferrooxidans cytochrome bc1 complex. These findings underline the genotypic heterogeneity within the A. ferrooxidans species. The results suggest that Iro transfers electrons from a cytochrome bc1 complex to a terminal oxidase, as proposed for the HiPIP in photosynthetic bacteria.

Abbreviations: DOP PCR, degenerate oligonucleotide primers PCR; HiPIP, high-potential iron–sulfur protein.

The GenBank/EMBL/DDJB accession numbers for the partial iro gene sequences from A. ferrooxidans strains CC1, ATCC 19859 and BRGM-1 are AJ621386–AJ621388 and the accession numbers of the iro locus from strains BRGM and ATCC 33020 are AJ621560 and AJ320262. Details of primers, an alignment of iro gene sequences from Fe-1 and ATCC 23270 and the sequence of the iro locus of ATCC 33020 are available as supplementary material with the online version of this paper.
gene in various pure strains of *A. ferrooxidans*, but were unable to detect it in any of them by PCR or Southern hybridization experiments. However, in the genome sequence of the type strain, ATCC 23270, a clearly related but distinct gene encoding an apparent HiPIP was found. To characterize this potential HiPIP from *A. ferrooxidans*, we overproduced it in *Escherichia coli*. Although attempts to express the *iro* gene from *A. ferrooxidans* strain Fe-1 in *E. coli* failed (Kusano et al., 1992), the potential HiPIP from strain ATCC 33020 was readily expressed in this neutrophilic bacterium. Its biochemical properties and its mode of transport to the periplasm of *E. coli* were established.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *A. ferrooxidans* ATCC 33020, ATCC 19859 and ATCC 23270 were obtained from the American Type Culture Collection. *A. ferrooxidans* BRGM was kindly provided by Dr D. Morin (Bureau de Recherches Géologiques et Minières, Orléans, France) (Battaglia-Brunet et al., 2002; Collinet & Morin, 1990). The isolation of *A. ferrooxidans* CC1 (Duquesne et al., 2003) has been described previously. *A. ferrooxidans* BRGM-1 was isolated from a BRGM iron culture on a solid medium (Liu et al., 2000). *E. coli* strain BL21(DE3) [F’ ompT hsdSB (argE388 mcrA76 dcm) lacIq lacZM15] was purchased from Novagen and strain TG1 [supE44 thi D(lac-proAB) F': traD36 proAB lacY1 Apir- lacZAM15] was used for phagemid propagation. *E. coli* strain TGC (TG1ΔattC) was kindly provided by Dr L.-F. Wu (Laboratoire de Chimie Bacteriennne, Marseille, France) (K. Gouffi and L.-F. Wu, unpublished results). Expression plasmids pET21 purchased from Novagen and pF191EH (Furste et al., 1986) were used for recombinant protein production. Phagemid Bluescript (SK +) was purchased from Stratagene. *A. ferrooxidans* was grown at 30 °C under oxic conditions on Fe<sup>2+ </sup>medium as already described (Duquesne et al., 2003). *E. coli* strains were grown on LB broth (Ausubel et al., 1992).

**DNA manipulations.** General DNA manipulations were performed according to Ausubel et al. (1992). Genomic DNA from *A. ferrooxidans* was prepared using the NucleoSpin Tissue kit from Macherey-Nagel, according to the manufacturer’s instructions for bacterial DNA extraction. The nucleotide sequences of the cloned fragments were determined from both strands by GENOME Express. For PCR amplifications, the oligonucleotides were obtained from Sigma-Genosys and Taq polymerase from Roche. Primers used in this study are given as supplementary material with the online version of this paper (Table S1).

**Degenerate oligonucleotide primers (DOP) PCR.** An alignment of nucleotide sequences was made between the *iro* genes of *A. ferrooxidans* strains Fe-1 and ATCC 23270 in order to determine the most conserved regions from which two degenerate oligonucleotides, *iro-degN* and *iro-degC*, were designed (see Supplementary Fig. S1 and Table S1). In a 10 μl reaction, 1 pmol of each primer was used and the PCR amplifications were carried out as follows: 2 min 30 s at 94 °C, 10 cycles at 94 °C for 30 s, 37 °C for 30 s and 72 °C for 30 s, 10 cycles at 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s, 10 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s and finally 2 min 30 s at 72 °C.

**Inverse PCR.** *A. ferrooxidans* ATCC 33020 genomic DNA was digested with *Alul* and *Taql* restriction enzymes (Biolabs). PCR was realized with the digested DNA as matrix according to Ochman et al. (1990) using the divergent oligonucleotides div-hip1 and div-hip2 deduced from the sequence determined by DOP PCR (Table S1).

**Cloning of DOP and inverse PCR fragments.** Amplification products were purified using Wizard PCR Preps (Promega). They were cloned in the EcoRV restriction site of the phagemid (SK +) Bluescript. Ligation products were used to transform *E. coli* strain TG1. White Ap<sup>R</sup> clones were selected on LB-Ap-Xgal-IPTG plates. The insert was amplified by PCR and sequenced with the universal primers T<sub>R</sub> and T<sub>B</sub>.

**Southern blotting.** Genomic DNA from *A. ferrooxidans* ATCC 33020 was digested with the restriction enzymes *KpnI*, *NcoI*, *PstI* and *SalI* (Biolabs). After electrophoresis, digested DNA was denatured and transferred by semi-dry capillary to a positively charged nylon membrane (Roche). A DIG-labelled probe corresponding to the *iro* gene was obtained by PCR with the oligonucleotides iro1 and airo3 (Table S1). Hybridization and washings were performed under high- and low-stringency conditions. Detection was accomplished by chemiluminescent reaction with CSPD [disodium 3-(4-methoxyxipirilo[1, 2-dioxetane-3,2'-5'-chloro)tricyclo[3.3.1.3<sup>2</sup>-yl] phenyl phosphate] as described by Roche.

**Pulsed field gel electrophoresis (PFGE).** PFGE was performed as previously described (Irazabal et al., 1997) on Spel-digested ATCC 33020 chromosomal DNA.

**Cloning of the ATCC 33020 iro gene.** The *iro<sub>33020</sub>* DNA corresponding to the precursor protein of strain ATCC 33020 was amplified by PCR from the genomic DNA of ATCC 33020 with the oligonucleotides hip-N*-Ban*HI and hip-C*-Xhol* (Table S1). The PCR fragments obtained were purified with Wizard PCR Preps and digested with *Ban*HI and *Xhol*. The purified fragments were ligated in the pET21(+) vector digested with *Ban*HI and *Xhol*. Cloning was done in *E. coli* strain T1G1, which lacks the T7 RNA polymerase structural gene and therefore cannot express the target gene. Screening was performed by PCR with oligonucleotides pET-T7 (5'-GGAGCCGTTAAACATTCGCCC-3') and T7-ter (5'-CGATC-CAATAAGCTGCCC-3'), which flank the multiple cloning site of the pET21 vector, and the oligonucleotides used to amplify the *iro<sub>33020</sub>* DNA. The sequence of the cloned fragments was checked. The recombinant plasmid pET21-*iro<sub>33020</sub>* was then introduced into *E. coli* BL21(DE3), which carries the T7 RNA polymerase structural gene under the control of the IPTG-inducible lacUV5 promoter.

The *iro<sub>33020</sub>* gene cloned in pET21 (see above) was amplified with primers pET-T7 and T7-ter. The PCR fragment obtained was blunted by T4 DNA polymerase, purified with Wizard PCR Preps and cloned in the vector pF191EH (Furste et al., 1986) previously digested with *SalI*. The sequence of the cloned fragments was checked. The pF191EH-*iro<sub>33020</sub>* plasmid was then introduced into *E. coli* strains T1G1 and TGC (TG1ΔattC).

**Protein analysis.** Periplasmic and spheroplastic fractions of *E. coli* were prepared as described previously (Bengrine et al., 1998). Protein concentration was estimated by the modified Bradford method (Bio-Rad protein assay) in 0·8 M NaOH according to the manufacturer’s guidelines. BSA was used as the standard. Equal amounts of proteins were heated at 100 °C for 5 min in Laemmli’s sample buffer and electrophoresed in 18% SDS-PAGE. Proteins were detected using the Phastgel Blue-R staining kit (Amersham Pharmacia Biotech).

Fe assays were performed according to the colorimetric method developed by Lovenberg et al., 1963). [4Fe–4S] cluster number per polypeptide was calculated by determining the absorption coefficient of the Iro protein using the *A<sub>580</sub>* and the protein concentration estimated by Bradford assays (Bartsch, 1978).
Purification of the recombinant Iro protein. *E. coli* strain BL21(DE3) carrying the pET21-*iro*<sub>33020</sub> plasmid was grown at 37 °C in LB with ampicillin (50 μg ml<sup>-1</sup>) to an OD<sub>600</sub> of 1-0 without induction. The recombinant His-tagged Iro protein was purified from the crude extract fraction using the His Trap kit (Amersham Pharmacia Biotech) according to the manufacturer’s guidelines. It was desalted using the HiTrap Desalting kit (Amersham Pharmacia Biotech) and dialysed against 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub> overnight at 4 °C with benzoylated dialysis tubing (Sigma-Aldrich).

**Western blotting.** Following SDS-PAGE, proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech) with the Mini Trans-Blot unit (Bio-Rad). The His-tagged Iro protein was identified by immunodetection with mouse anti-His antibodies (Amersham Pharmacia Biotech) and by using the Supersignal West Pico Trial kit (Pierce) as described in the manufacturer’s guidelines.

**Optical and EPR spectra.** Room-temperature spectra were recorded using a dual-wavelength DW2000 SLM-Aminco spectrophotometer. The concentration of HiPIP protein was determined using ε = 18 mM<sup>-1</sup> at 388 nm. EPR spectra were recorded at liquid helium temperature on a Bruker ESP 300E X-band spectrometer equipped with an Oxford Instruments liquid helium cryostat and temperature control system. See Fig. 4(b) for the EPR instrument settings.

**Electrochemical techniques.** Cyclic and square-wave voltammetry were carried out using an EG&G 273A potentiostat controlled by EG&G PAR M 270/250 software. A conventional three-electrode system was used consisting of a Metrohm Ag/AgCl/saturated NaCl reference electrode, a platinum auxiliary electrode and a membrane-pyrolytic graphite (MPG) (4 mm diameter rod) working electrode. Potentials versus the normal hydrogen electrode (NHE) have been obtained by adding 210 mV to the measured potentials (Bates, 1964). The membrane electrode technology was described in a previous paper (Haladjian et al., 1994). In such an electrode configuration, experiments require protein samples of only 1-2 μl, which were deposited on a square piece of dialysis membrane (Spectra/Por MWCO 6000–8000). The pyrolytic graphite electrode was then pressed against the membrane. A rubber ring was fitted around the electrode body so that the entrapped solution formed a uniform thin layer. Thus mounted, the MPG electrode was placed in the three-electrode cell containing only the buffer, which served as the supporting electrolyte. Because of the value of the membrane cut-off, imprisoned species were not able to diffuse through the membrane, in contrast to small ions (from/to the bulk solution in the cell). Prior to each experiment, the solutions were deoxygenated by bubbling high-purity nitrogen. All experiments were carried out at room temperature (about 23 °C) under a nitrogen atmosphere.

**RESULTS**

**Characterization of the *iro* gene in various strains of *A. ferrooxidans***

From the nucleotide sequence of the *iro* locus of strain Fe-1 (Kusano *et al*., 1992), two oligonucleotides, *iro1* and *airo3* (Table S1), were designed and used to perform PCR experiments on the genomic DNA of *A. ferrooxidans* strains ATCC 33020 and BRGM. Unexpectedly, no amplification product could be obtained when chromosomal DNA or cells of strain ATCC 33020 were used (Fig. 1). In contrast, amplification products of the expected size were obtained with the chromosomal DNA of strain BRGM (Fig. 1). The sequence of this PCR fragment is 100 % identical to the sequence of the *iro* locus from strain Fe-1, including the downstream end of *purA*, encoding adenylosuccinate synthase, and the intergenic region between these two genes (data not shown). The 390 bp DNA fragment obtained with oligonucleotides *iro1* and *airo3* (*iro*, *hip*<sub>9</sub> and *hip*<sub>10</sub> (*hip*) and *rusNM* and *RusCXhol* (*rus*). Lanes L, 1-kb Plus DNA ladder (Invitrogen).

In the genome sequence of the type strain of *A. ferrooxidans* (ATCC 23270; http://www.tigr.org/), no sequence related to *iro* was detected downstream of the *purA* gene, where the *iro* gene is located in strains Fe-1 and BRGM (Kusano *et al*., 1992; this study). On the other hand, a BLAST search revealed the existence of a gene encoding a protein showing 50.9 % identity and 73.6 % similarity to the Iro protein. The corresponding gene, *iro<sub>33020</sub>* was located downstream from the *petII* operon encoding the second cytochrome *bc<sub>1</sub>* complex of *A. ferrooxidans* (Brasseur *et al*., 2002; P. Bruscella, G. Levicán, J. Ratouchniki, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpublished results). These results could explain why the *iro* gene was not detected in strain ATCC 33020 by PCR or Southern hybridization experiments. Therefore, convergent degenerate oligonucleotides *iro-degN* and *iro-degC* were designed from conserved regions of the ATCC 23270 and Fe-1 *iro* genes, *iro<sub>23270</sub>* and *iro<sub>Fe-1</sub>* (Fig. S1 and Table S1), and used in DOP PCR experiments. Only one amplification product, the size of which corresponded to that expected for *iro<sub>23270</sub>* (271 bp), was obtained (data not shown). This fragment was cloned and sequenced. All the cloned fragments analysed had identical sequences and showed higher sequence similarity to *iro<sub>23270</sub>* (96 %) than to *iro<sub>Fe-1</sub>* (43-2 %). From this nucleotide sequence, convergent oligonucleotides (div-*hip*<sub>1</sub> and div-*hip*<sub>2</sub>; Table S1) were designed and used in inverse PCR on digested strain ATCC 33020 chromosomal DNA. Analysis of the sequence obtained (Supplementary Fig. S2) showed that the ATCC 33020 *iro* gene (*iro<sub>33020</sub>*) is located downstream from a gene orthologous to the ATCC 23270 *petC2* gene encoding the cytochrome *c<sub>1</sub>* of the second

![Fig. 1. PCR amplification of genomic DNA from *A. ferrooxidans* strains ATCC 33020, ATCC 23270 (type strain), ATCC 19859, CC1, BRGM-1 and BRGM with oligonucleotides *iro1* and *airo3* (*iro*, *hip*<sub>9</sub> and *hip*<sub>10</sub> (*hip*) and *rusNM* and *RusCXhol* (*rus*). Lanes L, 1-kb Plus DNA ladder (Invitrogen).](http://mic.sgmjournals.org)
cytochrome bc1 complex of *A. ferrooxidans* (Brasseur et al., 2002; P. Bruscella, G. Levica`n, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpubl. results).

We probed different *A. ferrooxidans* strains by PCR with oligonucleotides corresponding to the *iro* pet*C* locus (iro1 and airo3; Table S1) and to the *iro*33020 gene (hip9 and hip10; Table S1). Oligonucleotides rusNM and RusCXhol (Table S1), corresponding to the gene encoding rusticyanin, a protein so far described only in *A. ferrooxidans*, were used as controls. In addition to strain BRGM, we also tested the collection strains ATCC 19859, ATCC 23270 and ATCC 33020 and two private strains, which have been isolated on solid medium and shown unambiguously to belong to the *A. ferrooxidans* species by the sequences of the 16S rRNA gene and of the intergenic region between the 16S and 23S rRNA genes (Duquesne et al., 2003; CC1 (Duquesne et al., 2003) and BRGM-1, which is a BRGM clonal derivative (Liu et al., 2000). DNA was amplified with the oligonucleotides iro1 and airo3 (390 bp fragment) only from strain BRGM while, for all the strains tested, fragments of the expected size were obtained with rusNM and RusCXhol and with the hip9 and hip10 oligonucleotides (475 and 310 bp, respectively) (Fig. 1). The 310 bp DNA fragments were sequenced to confirm that they corresponded to *iro*33020 orthologous genes. Therefore, *iro*33020-orthologous genes are present in the six *A. ferrooxidans* strains we have tested, including strains BRGM and BRGM-1, whereas an *iro* petC-orthologous gene was detected only in strain BRGM and not in the BRGM-1 clonal derivative. Repeated attempts to isolate BRGM clonal derivatives containing the *iroBRGM* gene were unsuccessful.

**Description of the ATCC 33020 *iro* locus**

A potential ribosome-binding site, AAGGAG, was found seven nucleotides upstream from the translational start codon of the *iro*33020 gene (Fig. S2). No sequence with obvious similarity to the *E. coli* σ70-specific promoter and no obvious rho-independent transcriptional termination site were detected upstream from this gene. A potential hairpin structure (−16·1 kcal mol⁻¹; −67·4 kJ mol⁻¹) followed by a series of thymidines was found downstream from the *iro*33020 translational stop codon (Fig. S2) and could correspond to a transcriptional termination site. The nucleotide sequence corresponding to the *iro*33020 precursor is 95·6 % identical to the *iro*32720 gene and 56·2 % to the *iro* petC-1 and *iroBRGM* genes (Kusano et al., 1992; this study).

A typical signal sequence of the Tat secretion pathway (Bersk, 1996; Cristobal et al., 1999; Palmer & Berks, 2003) is present at the amino terminus of *iro*33020 (Fig. S2), characterized by (i) a relatively long signal peptide (48 of 106 aa), (ii) an extended positively charged region (23 aa) including, at the boundary, the motif S R R D M L K, where the underlined residues correspond to the Tat signal peptide consensus sequence, (iii) a small hydrophobic h-region (18 aa) rich in glycine and threonine and devoid of leucine residues, (iv) a 'potentially helix-breaking proline' at the end of this h-region and (v) a basic amino acid residue (histidine), known as 'sec-avoidance', in its hydrophilic c-region. The theoretical molecular mass deduced from the *iro*33020 amino acid sequence is 10·7 kDa for the precursor and 5·9 kDa for the mature protein. The isoelectric point of the mature *iro*33020 is estimated to be 8·3. The sequence of the deduced mature protein is 58·3 % identical (78·3 % similar) to the Iro protein from *A. ferrooxidans* strains Fe-1 (Kusano et al., 1992) and BRGM (this paper), 41 % identical (67·2 % similar) to the HiPIP protein from *Rhodopila globiformis* (Ambler et al., 1993) and 31·4 and 28·4 % identical (61·2 and 52·3 % similar) to the HiPIP from *Rhodocyclus tenuis* strains 2761 and 3761 (Tedro et al., 1979, 1985) (Fig. 2). The four cysteines which bind the iron atoms of the [4Fe–4S] cluster are conserved (Fig. 2). Therefore, the *iro*33020 protein belongs to the HiPIP family. Notably, its closest known relative, apart from *iro* petC-1 in *iroBRGM*, is the HiPIP from *Rhodopila globiformis*, which has a growth optimum of pH 5 (Ambler et al., 1993; Van Driessche et al., 2003).

An open reading frame encoding a putative polypeptide presenting high similarities to the cytochrome c1 of the bc1 complex encoded by the *petC* gene of *A. ferrooxidans* strains ATCC 23270 (Brasseur et al., 2002; P. Bruscella, G. Levica`n, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy,

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**Fig. 2.** Sequence comparisons of mature HiPIP. Alignment of the closest known relatives of the ATCC 33020 Iro protein: Iro from *A. ferrooxidans* strain ATCC 23270, Iro from *A. ferrooxidans* strains Fe-1 (Kusano et al., 1992) and BRGM (this study), HiPIP from *Rhodopila globiformis* (Ambler et al., 1993), HiPIP from *Rhodocyclus tenuis* 2761 (Tedro et al., 1985) and HiPIP from *Rhodocyclus tenuis* strain DSM 3761 (Tedro et al., 1979). Asterisks (*) indicate identical residues, colons (;) indicate strongly similar residues and dots (.) indicate weakly similar residues. The four conserved cysteines binding the [4Fe–4S] cluster are in bold.

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A. ferrooxidans ATCC 33020

A. ferrooxidans ATCC 23270

A. ferrooxidans Fe-1, BRGM

Rhodopila globiformis

Rhodocyclus tenuis 2761

Rhodocyclus tenuis DSM 3761

A. ferrooxidans ATCC 33020

A. ferrooxidans ATCC 23270

A. ferrooxidans Fe-1, BRGM

Rhodopila globiformis

Rhodocyclus tenuis 2761

Rhodocyclus tenuis DSM 3761

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unpublished results) was detected upstream from iro\textsubscript{33020} (Fig. S2). Downstream from iro\textsubscript{33020}, a putative gene preceded by a ribosome-binding site, GGAG, was located (Fig. S2). No similarity for the polypeptide it encodes was found in the databases. This open reading frame is also present downstream from iro\textsubscript{33270}. The genetic organization of the iro locus from strains ATCC 333020 and ATCC 23270 is therefore identical.

Characterization of recombinant His-tagged Iro\textsubscript{33020} protein produced in E. coli

Because of the relative low identity (58.3%) found between the mature Iro from two different strains (ATCC 333020 and Fe-1) of the same species, we decided to characterize Iro\textsubscript{33020}. To facilitate this characterization, we overproduced Iro\textsubscript{33020} in E. coli. For this purpose, the iro\textsubscript{33020} gene was cloned in the pET21 plasmid to produce the Iro\textsubscript{33020} precursor fused to a C-terminal hexahistidine tag as described in Methods. In IPTG-induced BL21(DE3)/pET21-iro\textsubscript{33020} cells, a polypeptide cross-reacting with anti-His antibodies of apparent molecular mass of about 12 kDa was detected, but not in control TG1/pET21-iro\textsubscript{33020} cells, which lack the T7 RNA polymerase structural gene (data not shown). The 12 kDa polypeptide was also detected, but in smaller amounts, in non-induced cells, presumably because of read-through of the T7 RNA polymerase promoter (data not shown). Fractionation revealed that this polypeptide is present mainly in the periplasm (Fig. 3a). In the spheroplast fraction of induced and non-induced cells, another polypeptide of about 16 kDa, probably the Iro\textsubscript{33020} precursor form, was detected (Fig. 3a). The recombinant Iro\textsubscript{33020} protein, now referred to as Iro\textsubscript{33020}-His\textsuperscript{Tag}, was purified on a nickel column from crude extracts of non-induced BL21(DE3)/pET21-iro\textsubscript{33020} cells as described in Methods. Two polypeptides, of 16 and 12 kDa, were obtained. The amino-terminal sequences of these polypeptides identified them as the precursor and mature forms of Iro\textsubscript{33020}. Both migrated in 18% polyacrylamide gels with an apparent molecular mass higher than those predicted from their amino acid sequence (10.7 and 6 kDa).

To determine the [4Fe–4S] cluster number per polypeptide, two approaches were followed: determination of the absorption coefficient of the purified Iro\textsubscript{33020}-His\textsuperscript{Tag} and Fe assays. In the first case, the absorption coefficient was estimated to be 16±1 M\textsuperscript{−1} cm\textsuperscript{−1}, in excellent agreement with the 16 M\textsuperscript{−1} cm\textsuperscript{−1} absorption coefficient of the HiPIP from Allochromatium vinosum (Bartsch, 1978; Heering et al., 1995), which contains a single [4Fe–4S] cluster (Carter et al., 1974). The total iron content of the purified Iro\textsubscript{33020}-His\textsuperscript{Tag}, determined by the colorimetric method of Lovenberg et al. (1963), was estimated to be 4.4 μmol (μmol protein\textsuperscript{−1}). The Iro\textsubscript{33020}-His\textsuperscript{Tag} protein contains therefore a single [4Fe–4S] cluster, in agreement with the amino acid sequence, in which a single [4Fe–4S] cluster binding site was observed (see above and Fig. 2).

Optical spectra of purified Iro\textsubscript{33020}-His\textsuperscript{Tag} showed changes in absorbance depending upon oxidation or reduction states. The optical spectrum of reduced Iro\textsubscript{33020}-His\textsuperscript{Tag} exhibited a significant decrease in absorbance in the region of 450–550 nm compared with the oxidized Iro\textsubscript{33020}-His\textsuperscript{Tag} (Fig. 4a), and the oxidized minus reduced spectrum exhibited a characteristic maximum absorption at 490 nm (Fig. 4a inset), suggesting that Iro\textsubscript{33020}-His\textsuperscript{Tag} behaves as a correctly folded [4Fe–4S]-cluster-containing protein. A purity ratio (A\textsubscript{278}/A\textsubscript{388}) of 1.9 was obtained for the reduced HiPIP. The sample showed no contaminants that stained with Coomassie blue on SDS-PAGE (data not shown). As observed with other HiPIPs, the reduced state of Iro\textsubscript{33020}-His\textsuperscript{Tag} exhibited no EPR signal (Fig. 4b). Upon oxidation by hexachloroiridate (IrCl\textsubscript{6}), a typical HiPIP axial EPR signal characterized by g = 2.043, g = 2.115 appeared (Fig. 4b), indicating the presence of the [4Fe–4S] cluster.

![Fig. 3. Localization of the recombinant Iro protein in E. coli.](http://mic.sgmjournals.org)
These results confirm that the Iro33020 of *A. ferrooxidans* belongs to the HiPIP family and that, when produced in *E. coli*, this protein has incorporated a $[4\text{Fe}–4\text{S}]$ cluster into a folded native form that is correctly processed and translocated to the periplasm. The electrochemical behaviour of Iro33020-HisTag was investigated. Typical cyclic and square-wave voltammograms obtained at the MPG electrode are shown in Fig. 5(a, b) for a 40 μM Iro33020-HisTag solution entrapped between the dialysis membrane and the pyrolytic graphite electrode (see Methods). A quasi-reversible behaviour was obtained and the redox potential either measured as the square-wave voltammogram peak potential (Fig. 5b) or calculated as the mean of the cathodic and anodic cyclic voltammogram peaks (Fig. 5a) was $510 \pm 5$ mV at pH 7.4. This very high redox potential confirms that Iro33020 belongs to the HiPIP family. To our knowledge, this is one of the highest redox potentials described for HiPIP $[4\text{Fe}–4\text{S}]$ clusters (Capozzi *et al*., 1998). It has been suggested previously that the major determinant for the variation of redox potential within HiPIP series (from 100 to 500 mV) was the difference in charged residues. The more positive the charge of the protein, the higher the redox potential of the cluster. In this work, a good correlation between the global charge of the
protein and its high redox potential is obtained, since the pI of Iro33020 is estimated to be 8.3.

The pH-dependence of the redox potential was investigated in the pH range 2–9 (Fig. 5c). It is noteworthy that a well-shaped and stable electrochemical signal was obtained, suggesting good stability of the protein over a large pH range. This result is not unexpected since the pH of the A. ferrooxidans periplasm, where Iro33020 is localized, is low. The redox potential of HiPIP decreased linearly with a slope of 15 mV per pH unit in the pH range 3–5, but remained constant at a lower and higher pH. At the very low pH of 2, the redox potential of Iro33020 reached 550 mV. Note that the same evolution of redox potential with pH has been observed for rusticyanin, a periplasmic blue-copper protein (Haladjian et al., 1993), and cytochrome c552 (Haladjian et al., 1994), from the same micro-organism.

The recombinant Iro33020-HisTag protein is translocated in the E. coli periplasm by the Tat system

The Iro33020 protein has a typical signal sequence of the Tat secretion pathway (see above). To test whether this signal sequence is recognized by the Tat pathway of the neutrophilic E. coli, iro33020 expression was compared between a wild-type and a tat-deficient mutant of E. coli. Because we were unable to construct a BL21(DE3) tat mutant, we cloned iro33020 in the pLFI19EH expression vector and introduced the recombinant plasmid (pLFI19EH-iro33020) in the isogenic TG1 and TGC (ΔtatC) strains of E. coli. The mature form of the Iro33020 protein was detected by immunodetection with antibodies directed against the His-tag in the periplasmic fraction of the wild-type E. coli carrying the construct pLFI19EH-iro33020 (Fig. 3b). In contrast, no mature form of Iro33020 was detected in the periplasmic fraction of the ΔtatC mutant of E. coli. Furthermore, a small amount of the precursor form of Iro33020 was detected in the spheroplastic fraction of the wild-type and of the ΔtatC mutant after a longer exposure (Fig. 3c). These results indicated that, in spite of the periplasm pH difference, the A. ferrooxidans Iro33020 protein was processed and translocated to the periplasm via the Tat secretion pathway of E. coli.

DISCUSSION

A HiPIP-encoding gene has been detected in five pure strains of A. ferrooxidans. The nucleotide sequence of this gene presents 56.2% identity with the iro gene encoding a HiPIP previously characterized in A. ferrooxidans strain Fe-1 (Kusano et al., 1992).

When the iro33020 gene was expressed in E. coli, the recombinant Iro33020-HisTag protein had all the expected properties of a HiPIP with a correctly incorporated [4Fe–4S] cluster. As shown by optical and EPR spectra analyses (Fig. 4), the recombinant Iro33020-HisTag protein contains a typical HiPIP [4Fe–4S] cluster. Furthermore, the redox potential of the protein was high (+550 mV at low pH), as expected for a HiPIP from an acidophile (Fig. 5). Because recombinant HiPIPs produced in E. coli exhibit properties very similar to the native proteins (Agarwal et al., 1993; Eltis et al., 1994; Brüser et al., 1998; Caspersen et al., 2000), the described properties of the recombinant Iro33020-HisTag produced in E. coli likely reflect those of the native Iro33020 protein. Indeed, they are similar to those of IroFe-1 and IroBRGM (Fukumori et al., 1988; Yamanaka & Fukumori, 1995; Cavazza et al., 1995).

In spite of the fact that E. coli is a neutrophile and encodes no apparent HiPIP (Blattner et al., 1997), the correctly processed HiPIP Iro from the acidophilic A. ferrooxidans can be successfully produced in this bacterium. In E. coli, complex systems are required for the assembly of iron–sulfur clusters (Takahashi & Nakamura, 1999; Takahashi & Tokumoto, 2002). Putative genes involved in the formation of iron–sulfur centres were identified in the genome sequence of A. ferrooxidans (Valdés et al., 2003). Nevertheless, reconstitution of the [4Fe–4S] cluster in vitro into a HiPIP precursor has been reported (Brüser et al., 2003), suggesting that loading of the iron–sulfur centre in HiPIP may also take place without accessory proteins.

The production of periplasmic redox holoproteins from the acidophilic A. ferrooxidans in the neutrophilic E. coli has presented some experimental problems. Rusticyanin, a blue-copper protein, has been produced in the cytoplasm (Casimiro et al., 1995) or in the periplasm (Bengrine et al., 1998) but, in both cases, the copper was not incorporated and reconstitution in vitro was required. Similarly, copper insertion in subunit II of the aa3-type cytochrome oxidase from A. ferrooxidans did not occur when produced in the periplasm of E. coli (J. A. DeMoss, K. Lund, J. Ratouchniak and V. Bonnefoy, unpublished results). In the case of cytochromes c, a plasmid constitutively expressing the E. coli ccm operon encoding the cytochrome c maturation system was required in order to obtain holocytochromes (Appia-Ayme, 1998). In all these examples, the recombinant protein was correctly translocated to the periplasm of E. coli, however. Why was no cofactor loaded in these proteins, while Iro33020-HisTag had its [4Fe–4S] cluster incorporated? The simplest explanation is that cytochromes c, rusticyanin and subunit II of the aa3-type cytochrome oxidase are translocated by the Sec system, while Iro33020-HisTag is translocated by the Tat pathway. In the former case, the cofactor is inserted in the periplasm and, in the latter case, it takes place in the cytoplasm before translocation (Palmer & Berks, 2003). Although there is a significant difference in the pH of the periplasm between E. coli (pH 6–7) and A. ferrooxidans (pH 1.5–3), the pH of the cytoplasm of the two organisms is approximately the same (see Ingledew, 1982 and references therein). We have shown in this paper that translocation of the Iro33020-HisTag protein is Tat-dependent in E. coli, in spite of the fact that the cytoplasmic membranes of A. ferrooxidans and E. coli are facing different pH. First, it has a characteristic twin-arginine signal
sequence. Second, recombinant Iro33020-HisTag protein failed to be translocated to the periplasm in a tar mutant of E. coli (Fig. 3b). These data indicate furthermore that the twin-arginine signal sequence of Iro from the acidophile *A. ferrooxidans* is recognized by the Tat system of the neutrophile *E. coli*. Computer searches have revealed a locus containing *tatA*, *tatB* and *tatC* genes (Wu et al., 2000), a paralogous *tata* gene and two *tadT* genes in the genome of *A. ferrooxidans* ATCC 23270.

The *iro*33270 gene is present not only in *A. ferrooxidans* strain ATCC 33020, but also in ATCC 19859 and in two private pure strains. The genetic organization of the *iro* locus differs depending on the strain: *iro* is located downstream from *purA* in strains BRGM and Fe-1 (Kusano et al., 1992; this study) and upstream from a putative leucyl-tRNA gene in strain Fe-1 (Kusano et al., 1992), while it is located downstream from the *petII* operon encoding the second *bc*1 complex of *A. ferrooxidans* in ATCC 23270 and ATCC 19859 (Brasseur et al., 2002; P. Bruscella, G. Levica`n, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpublished results) and ATCC 33020 (this study). Downstream, no tRNA gene was detected, but instead a putative gene encoding a protein with no similarity with proteins in the databases was found. The amino acid sequence of the mature Iro33020 has 58-3 % identity to the mature IroFe-1 while, for example, the mature rusticyanin from strain Fe-1 (Kusano et al., 1995). Therefore, we can wonder whether the *iroFe-1* and *iro33020* are orthologous genes or encode HiPIPs with different functions. One possibility we cannot exclude is that IroBRGM was not purified from *A. ferrooxidans* but from another micro-organism, as strain BRGM, from which the *iroBRGM* gene and IroBRGM protein have been analysed (Cavazza et al., 1995 and this paper), is in fact a bacterial consortium (Battaglia-Brunet et al., 2002; Collinet & Morin, 1990). However, *A. ferrooxidans* strains have been reported by several authors to be physiologically and genomically diverse (Leduc & Ferroni, 1994; Harrison, 1982, 1984) and to diverge in different phylogenetic groups (Goebel & Stackebrandt, 1994; Karavaiko et al., 2003; Novo et al., 1996; Selenksa-Pobell et al., 1998). In this case, the simplest explanation is that strains Fe-1 and BRGM belong to a different group from the *A. ferrooxidans* strains analysed in this paper. The phylogenetic tree constructed from HiPIP amino acid sequences (Van Driessche et al., 2003; data not shown) shows that IroFe-1, Iro33270 and Iro33020 are very close and supports the second hypothesis, according to which these two proteins are encoded by orthologous genes of *A. ferrooxidans* strains belonging to two different phylogenetic groups. Furthermore, the recombinant Iro33020-HisTag produced in *E. coli* has properties similar to those of IroFe-1 and IroBRGM (Fukumori et al., 1988; Yamanaka & Fukumori, 1995; Cavazza et al., 1995).

Based on the current results, does Iro play the role proposed by Fukumori et al. (1988) and Yamanaka & Fukumori (1995) or does it have another function? All the HiPIPs for which a role has been determined, except Iro, act as an electron shuttle between the *bc*1 complex and either the photosynthetic reaction centre complex (Schoepp et al., 1995; Hochkoeppler et al., 1995, 1996; Menin et al., 1997; Nagashima et al., 2002; Verméglio et al., 2002) or a terminal oxidase (Bonora et al., 1999; Pereira et al., 1999). Yamanaka and co-workers (Fukumori et al., 1988; Yamanaka & Fukumori, 1995) have proposed that the Iro protein is the first electron carrier in the ferrous iron respiratory chain, from which its name is derived. The high molecular mass cytochrome Cyc2 is a better candidate to play this role, as already discussed in our previous papers (Appia-Ayme et al., 1999; Yarzábal et al., 2002). Indeed, this cytochrome is an outer-membrane protein (Yarzábal et al., 2002) and, because ferrous oxidation has been inferred to take place outside the cell, it is a more likely candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen than the periplasmic HiPIP. Furthermore, the *cyc2* gene encoding this cytochrome belongs to the same operon as the genes encoding the cytochrome *c*4 Cyc1, rusticyanin and the *aa3*-type cytochrome oxidase (Appia-Ayme, 1998; Appia-Ayme et al., 1999), which have been proposed to be involved in ferrous iron oxidation (Yarzábal et al., 2004). Interestingly, the *petII*-paralogous operon encoding the second cytochrome *bc*1 complex of *A. ferrooxidans* (Brasseur et al., 2002) has been detected upstream from *iro* in several strains (this study; P. Bruscella, G. Levica`n, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpublished results). Furthermore, preliminary results have shown that *iro* belongs to the *petII* operon in strains ATCC 33020 and ATCC 19859 (P. Bruscella, G. Levica`n, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpublished results). Interestingly, the Iro redox potential (550 mV) determined in this paper is between that proposed for the *bc*1 complex of *A. ferrooxidans* (Elbehti et al., 1999) and that for the terminal oxidase (Kai et al., 1992). Therefore, we suggest 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 are other known HiPIP (Bonora et al., 1999; Pereira et al., 1999). We therefore propose to refer to this HiPIP protein as Hip, until its physiological role is determined, and to the corresponding gene as *hip*.

**ACKNOWLEDGEMENTS**

We owe special thanks to Dr P. Bianco and Dr W. Nitschke (Laboratoire de Bioénergétique et Ingénierie des Protéines, IBSM, Marseille, France) and Dr A. Yarzábal and Dr C. Appia-Ayme (Laboratoire de Chimie Bactérienne, IBSM, Marseille, France) for helpful discussions. We thank Professor J. DeMoss and A. Manvell for critical reading of the manuscript. We thank Professor B. Guigliarelli and co-workers for access to the EPR machine. We are grateful to Mrs E. Gonzalez Partida for her help in PFGE experiments. We acknowledge Dr D. Morin (Bureau de Recherches Géologiques et Minières, Orléans, France) and Dr L.-F. Wu (Laboratoire de Chimie Bactérienne, IBSM, Marseille, France) for kindly providing *A. ferrooxidans* strain.
BRGM and *E. coli* strain TGC, respectively. Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *A. ferrooxidans* was accomplished with support from the US Department of Energy. We are grateful to R. Lebrun from the IBSM Protein Sequencing Unit (Marseille, France) for performing the N-terminal sequence determination. This study was partly supported by a Picasso program award to R.A. and V.B.

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