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.

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INTRODUCTION

One of the most studied bacteria that thrives in acidic mine drainage is Acidithiobacillus ferrooxidans, formerly Thiobacillus ferrooxidans (Kelly & Wood, 2000; Leduc & Ferroni, 1994). It is characterized as a Gram-negative, acidophilic chemolithoautotrophic that obtains energy for its growth mainly from the oxidation of ferrous iron (Fe2+) or reduced sulfur compounds (Ingledew, 1982). Attempts to understand such an energetic metabolism, and especially the oxidation of Fe2+, have been initiated, leading to the proposition of several respiratory chains involving various redox proteins that have been identified and characterized in A. ferrooxidans (Fukumori et al., 1988; Blake & Shute, 1994; Yamanaka & Fukumori, 1995; Giudici-Orticoni et al., 1997, 2000; Appia-Ayme, 1998; Appia-Ayme et al., 1999). Among them, a high-potential iron–sulfur protein (HiPIP) designated Iro, for iron-oxidizing enzyme, was characterized in A. ferrooxidans strains Fe-1 (Fukumori et al., 1988; Kusano et al., 1992) and BRGM (Cavazza et al., 1995) and the corresponding iro gene was also cloned and studied from strain Fe-1 (Kusano et al., 1992). The Iro protein was proposed to be the first electron acceptor in several alternative models of electron transfer chain between Fe2+ and oxygen (Fukumori et al., 1988; Yamanaka et al., 1995). Based on genetic and subcellular localization studies with strain ATCC 33020, we proposed a model, not involving Iro, in which the electrons are passed from Fe2+ to oxygen through a set of redox proteins expressed from an operon (Appia-Ayme, 1998; Appia-Ayme et al., 1998, 1999; Bengrine et al., 1998; Yarzábal et al., 2002) that is induced in Fe2+-grown cells (Yarzábal et al., 2004). To investigate the possible role of Iro, we probed for the presence of the iro gene encoding a putative high-potential iron–sulfur protein (HiPIP) from the strictly acidophilic and 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/DDBJ 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.

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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 Miérères, Orléans, France) (Bataggia *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*omp1* hsdS*B* (acl857 ind1 Sam7 nin5 lacUV5-T7 gene)], purchased from Novagen and strain TG1 [supE thi dcm *lac-proAB* F*: traD36 proAB lacI*Δ* lac*ZAM15] was purchased for phagemid propagation. *E. coli* strain TG1 (TG1*Δatc*) was kindly provided by Dr L.-F. Wu (Laboratoire de Chimie Bactérienne, Marseille, France) (K. Gouffi and L.-F. Wu, unpublished results). Expression plasmids pET21 purchased from Novagen and pJF119EH (Furste *et al.*, 2003) were kindly provided by Dr L.-F. Wu (Laboratoire de Chimie Bactérienne, Marseille, France) (K. Gouffi and L.-F. Wu, unpublished results). Expression plasmids pET21 purchased from Novagen and pJF119EH (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*2+* 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 GENEMARK 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 *Alu*I and *Taq*I restriction enzymes (Biolabs). PCR was realized with the religated 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 Prep (Promega). They were cloned in the EcoRI restriction site of the phagemid (SK*) Bluescript. Ligation products were used to transform *E. coli* strain TG1. White Ap*c* clones were selected on LB-Ap-Xgal-IPTG plates. The insert was amplified by PCR and sequenced with the universal primers T3 and T7.

**Southern blotting.** Genomic DNA from *A. ferrooxidans* ATCC 33020 was digested with the restriction enzymes KpnI, NcoI, PstI and SfiI (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-methoxyisopropyl-1, 2-dioxetane-3,2’-5’-chloro) tricyclo[3,3.1.1,3’] decan]-4-yl phenyl phosphate] as described by Roche.

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

**Cloning of the ATCC 33020 *iro* gene.** The *iro* gene 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-BamHI and hip-C-Xhol (Table S1). The PCR fragments obtained were purified with Wizard PCR Prep and digested with *BamHI* and *Xhol*. The purified fragments were ligated in the pET21 (+) vector digested with *BamHI* and *Xhol*. Cloning was done in *E. coli* strain TG1, 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*-GATACGCGGATCCACTTCCCCTC-3*) and T7-ter (5*-CGATGATATCGGAGGCTGC-3*), which flank the multiple cloning site of the pET21 vector, and the oligonucleotides used to amplify the *iro* gene. DNA fragment of the cloned fragments was confirmed. The recombinant plasmid pET21-*iro* 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* gene cloned in pET21 (see above) was amplified with primers pET-T7 and T7-ter. The PCR fragment obtained was blunt-ended by T4 DNA polymerase, purified with Wizard PCR Prep and cloned in the vector pJF119EH (Furste *et al.*, 1986) previously digested with *SmaI*. The sequence of the cloned fragments was confirmed. The pJF119EH-*iro* plasmid was then introduced into *E. coli* strains T1 and TGC (TG1*Δatc*).

**Protein analysis.** Periplasmic and spheroplast 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 poly peptide was calculated by determining the absorption coefficient of the Iro protein using the *A*405 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<sup>33020</sup> 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 HiTrap 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 voltammery 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-pyrolitic 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 pyrolitic 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 was used as a probe for Southern blotting with conventional electrophoresis and PFGE using chromosomal DNA of strain ATCC 33020. No hybridization was detected, even under low-stringency conditions (data not shown).

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>, is 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. Levica, J. Ratouchniak, 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>33020</sub> (96%) than to iro<sub>Fe-1</sub> (43.2%). From this nucleotide sequence, divergent oligonucleotides (div-hiP1 and div-hiP2; 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
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, unpublished results).

We probed different *A. ferrooxidans* strains by PCR with oligonucleotides corresponding to the iroFε1 locus (iro1 and airo3; Table S1) and to the iro33020 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 iro33020-orthologous genes. Therefore, iro33020-orthologous genes are present in the six *A. ferrooxidans* strains we have tested, including strains BRGM and BRGM-1, whereas an iroFε1-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 iro33020 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 iro33020 translational stop codon (Fig. S2) and could correspond to a transcriptional termination site. The nucleotide sequence corresponding to the iro33020 precursor is 95·6% identical to the iro33270 gene and 56·2% to the iroFε1 and iroBRGM genes (Kusano et al., 1992; this study). A typical signal sequence of the Tat secretion pathway (Berks, 1996; Cristobal et al., 1999; Palmer & Berks, 2003) is present at the amino terminus of Iro33020 (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 Iro33020 amino acid sequence is 10·7 kDa for the precursor and 5·9 kDa for the mature protein. The isoelectric point of the mature Iro33020 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 glabriformis* (Ambler et al., 1993) and 31·4 and 28·4% identical (61·2 and 52·3% similar) to the HiPIP from *Rhodococcus 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 Iro33020 protein belongs to the HiPIP family. Notably, its closest known relative, apart from IroFe-1 and IroBRGM, is the HiPIP from *Rhodopila glabriformis*, 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 *c*1 of the bc1 complex encoded by the petC2 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, unpublished results). The 310 bp DNA fragments from strain ATCC 23270 were obtained with rusNM and RusCXhol using the oligonucleotides hip9 and hip10 (475 and 310 bp, respectively) (Fig. 1). The 310 bp DNA fragments were sequenced to confirm that they corresponded to iro33020-orthologous genes. Therefore, iro33020-orthologous genes are present in the six *A. ferrooxidans* strains we have tested, including strains BRGM and BRGM-1, whereas an iroFε1-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.

**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 glabriformis* (Ambler et al., 1993), HiPIP from *Rhodococcus tenuis* 2761 (Tedro et al., 1985) and HiPIP from *Rhodococcus tenuis* 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.
unpublished results) was detected upstream from \(iro_{33020}\) (Fig. S2). Downstream from \(iro_{33020}\), 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 \(iro_{33270}\). The genetic organization of the \(iro\) locus from strains ATCC 33020 and ATCC 23270 is therefore identical.

**Characterization of recombinant His-tagged \(Iro_{33020}\) protein produced in *E. coli***

Because of the relative low identity (58.3%) found between the mature Iro from two different strains (ATCC 33020 and Fe-1) of the same species, we decided to characterize \(Iro_{33020}\). To facilitate this characterization, we overproduced \(Iro_{33020}\) of the same species, we decided to characterize the mature \(Iro\) from two different strains (ATCC 33020 and ATCC 23270). Because of the relative low identity (58.3%) found between \(Iro_{33020}\) and \(Iro_{23270}\), the genetic organization of the \(iro\) locus from strains ATCC 33020 and ATCC 23270 is therefore identical.

**Iro33020** protein produced in *E. coli*

Characterization of recombinant His-tagged \(Iro_{33020}\) protein produced in *E. coli*

To determine the [4Fe–4S] cluster number per polypeptide, two approaches were followed: determination of the absorption coefficient of the purified \(Iro_{33020}\)-HisTag and Fe assays. In the first case, the absorption coefficient was estimated to be \(16 ± 1 \text{ mM}^{-1} \text{ cm}^{-1}\), in excellent agreement with the \(16 \text{ mM}^{-1} \text{ cm}^{-1}\) 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 \(Iro_{33020}\)-HisTag, determined by the colorimetric method of Lovenberg *et al.* (1963), was estimated to be \(4.4 \mu\text{mol (}\mu\text{mol protein})^{-1}\). The \(Iro_{33020}\)-HisTag 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 \(Iro_{33020}\)-HisTag showed changes in absorbance depending upon oxidation or reduction states. The optical spectrum of reduced \(Iro_{33020}\)-HisTag exhibited a significant decrease in absorbance in the region of 450–550 nm compared with the oxidized \(Iro_{33020}\)-HisTag (Fig. 4a), and the oxidized minus reduced spectrum exhibited a characteristic maximum absorption at 490 nm (Fig. 4a inset), suggesting that \(Iro_{33020}\)-HisTag behaves as a correctly folded [4Fe–4S]-cluster-containing protein. A purity ratio \((A_{278}/A_{388})\) 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 \(Iro_{33020}\)-HisTag exhibited no EPR signal (Fig. 4b). Upon oxidation by hexachloroiridate (IrCl₆), 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*. (a) BL21/pET21-iro\(_{33020}\) cells were induced with 0.1 mM IPTG for 3 h at 37°C. Periplasmic and spheroplast fractions were electrophoresed on 18% SDS-PAGE, transferred to a PVDF membrane and analysed by immunodetection with anti-His antibodies. (b) and (c) Western immunoblots of the periplasm and spheroplast fractions from non-induced TG1/pJF119EH-iro\(_{33020}\) (wt) and TGC/pJF119EH-iro\(_{33020}\) (ΔtatC) cells with antiserum raised against the His-tag. The recombinant Hip protein purified from strain BL21/pET21-iro\(_{33020}\) was included as a control on the right. Each lane was loaded with 1 μg protein, except for the periplasmic fraction of TGC/pJF119EH-iro\(_{33020}\) (2 μg) and the purified recombinant Iro\(_{33020}\) protein (0.5 μg). Blots were exposed for 30 s (b) and 15 min (c).
These results confirm that the Iro\textsubscript{33020} of \textit{A. ferrooxidans} belongs to the HiPIP family and that, when produced in \textit{E. coli}, this protein has incorporated a [4Fe–4S] cluster into a folded native form that is correctly processed and translocated to the periplasm.

The electrochemical behaviour of Iro\textsubscript{33020}-HisTag was investigated. Typical cyclic and square-wave voltammograms obtained at the MPG electrode are shown in Fig. 5(a, b) for a 40 \textmu{}M Iro\textsubscript{33020}-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 ± 5 mV at pH 7.4. This very high redox potential confirms that Iro\textsubscript{33020} belongs to the HiPIP family. To our knowledge, this is one of the highest redox potentials described for HiPIP [4Fe–4S] clusters (Capozzi \textit{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 3.5–5, 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 pUF19EH expression vector and introduced the recombinant plasmid (pUF19EH-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 pUF19EH-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; Elitis 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 aao-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 aao-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 6–5) 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 Iro<sub>33020</sub>-HisTag protein failed to be translocated to the periplasm in a tar mutant of <i>E. coli</i> (Fig. 3b). These data indicate furthermore that the twin-arginine signal sequence of Iro from the acidophile <i>A. ferrooxidans</i> is recognized by the Tat system of the neutrophile <i>E. coli</i>. Computer searches have revealed a locus containing tatA, tatB and tatC genes (Wu et al., 2000), a paralogous tatA gene and two tatD genes in the genome of <i>A. ferrooxidans</i> ATCC 23270.

The <i>iro</i><sub>33270</sub> gene is present not only in <i>A. ferrooxidans</i> strain ATCC 33020, but also in ATCC 19859 and in two private pure strains. The genetic organization of the <i>iro</i> locus differs depending on the strain: <i>iro</i> is located downstream from <i>purA</i> 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 <i>bc</i><sub>1</sub> complex of <i>A. ferrooxidans</i> in ATCC 23270 and ATCC 19859 (Brasseur et al., 2002; P. Bruscella, G. Leviscān, 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 Iro<sub>33020</sub> has 58-3 % identity to the mature Iro<sub>Fe-1</sub> while, for example, the mature rusticyanin from strain ATCC 33020 has 92-9 and 92-3 % identity to the mature rusticyanin from strains Fe-1 and BRGM, respectively (Guigliarelli et al., 1995). Therefore, we can wonder whether the <i>iro</i><sub>Fe-1</sub> and <i>iro</i><sub>33020</sub> are orthologous genes or encode HiPIPs with different functions. One possibility we cannot exclude is that Iro<sub>BRGM</sub> was not purified from <i>A. ferrooxidans</i> but from another micro-organism, as strain BRGM, from which the <i>iro</i><sub>BRGM</sub> gene and Iro<sub>BRGM</sub> 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, <i>A. ferrooxidans</i> strains have been reported by several authors to be physiologically and genomically diverse (Leduc & Ferroni, 2002; Collinet & Morin, 1990). Nevertheless, the <i>petII</i>-paralogous operon encoding the second cytochrome <i>bc</i><sub>1</sub> complex of <i>A. ferrooxidans</i> (Brasseur et al., 2002) has been detected upstream from <i>iro</i> in several strains (this study; P. Bruscella, G. Leviscān, J. Ratouchniak, E. Jedlicki, D. S. Holmes and V. Bonnefoy, unpublished results). Furthermore, preliminary results have shown that <i>iro</i> belongs to the <i>petII</i> operon in strains ATCC 33020 and ATCC 19859 (P. Bruscella, G. Leviscān, 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 <i>bc</i><sub>1</sub> complex of <i>A. ferrooxidans</i> (Elbehiti 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 <i>bc</i><sub>1</sub> 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 <i>hip</i>.

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