Characterization of a flavocytochrome that is induced during the anaerobic respiration of Fe³⁺ by *Shewanella frigidimarina* NCIMB400

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A 63.9 kDa periplasmic tetrahaem flavocytochrome *c*₃, designated Ifc₃, was found to be expressed in *Shewanella frigidimarina* NCIMB400 grown anaerobically with ferric citrate or ferric pyrophosphate as the sole terminal electron acceptor, but not in anaerobic cultures of the bacterium with other respiratory substrates. Ifc₃ was purified to homogeneity and revealed by biochemical, spectroscopic and primary structure analyses to contain four low-spin bis-His-ligated *c*₃-haems, with midpoint reduction potentials of −73, −141, −174 and −259 mV. A low-potential flavin was present in the form of non-covalently bound FAD; the protein possessed a unidirectional fumarate reductase activity. Disruption of the chromosomal gene encoding

Ifc₃, *ifcA*, did not lead to a significant change in the rate of Fe³⁺ reduction in batch culture. However, during such growth the Ifc₃-deficient mutant produced both a 35 kDa periplasmic *c*-type cytochrome and a 45 kDa membrane-associated *c*-type cytochrome at markedly higher levels than did the parent strain. Nucleotide sequencing data from directly upstream of *ifcA* indicated the presence of an open reading frame encoding a putative outer-membrane β-barrel protein of 324 amino acid residues.

Key words: bacteria, ferric reductase, flavin, haem, iron.

INTRODUCTION

Bacterial respiratory Fe³⁺ reduction can be defined as the action of Fe³⁺ as the terminal electron acceptor in an energy-conserving electron transport pathway, resulting in the extracellular accumulation of Fe²⁺. The process is of widespread environmental significance, influencing several biogeochemical cycles and affecting soil properties and plant growth [1]. However, the current interest in Fe³⁺-respiring micro-organisms arises from their potential applications in bioremediation. Complete oxidation of the aromatic hydrocarbons benzene and toluene to CO₂ is coupled to Fe³⁺ reduction by strict anaerobes such as *Geobacter metallireducens*; petroleum components might therefore be degraded by the bacteria in anoxic zones [2]. Furthermore, Fe³⁺-respirers tend to be able to reduce highly soluble U^{VI} and Tc^{VII} species, resulting in the deposition of these metals as insoluble lower-valence forms and thus effecting their removal from contaminated waters [3].

A number of phylogenetically diverse micro-organisms isolated from a variety of habitats are capable of respiratory Fe³⁺ reduction [4]. Studies on the biochemistry of the process are limited, and have focused largely on the Gram-negative, non-fermentative, highly versatile facultative anaerobe *Shewanella putrefaciens* [5]. This bacterium can obtain energy for growth by respiring Fe³⁺ with H₂, formate, lactate or pyruvate as the electron donor [6,7]. The cation has been supplied as poorly soluble ferric oxyhydroxides or in solution complexed by the ligand citrate. Other terminal electron acceptors utilized by *S.*

putrefaciens include Mn^{IV} oxides [8] and elemental sulphur [9]. Mutagenesis studies have revealed a membrane-anchored tetrahaem cytochrome *c*, CymA [10], to be a component of the electron transport pathway to Fe³⁺ in *S. putrefaciens* MR-1. On the basis of primary structure analysis [10], CymA is a member of the NapC/NirT family found in a range of facultatively and obligately anaerobic bacteria. These proteins are most probably involved in mediating electron transfer between membrane-entrapped quinol and periplasmic redox proteins [11]. CymA is also required for the anaerobic metabolism of *S. putrefaciens* MR-1 with other terminal electron acceptors, including nitrate, fumarate and Mn^{IV} [10].

No terminal Fe³⁺-reducing enzymes have yet been irrefutably identified in *S. putrefaciens*. An Fe³⁺ reductase activity has, however, been located at the outer membrane of strain MR-1 of the bacterium [12]; electron transfer from primary dehydrogenases to the cell exterior therefore seems likely to occur. This would allow Fe³⁺ species that are unable to access the periplasmic compartment, such as insoluble ferric oxyhydroxides, to act as respiratory substrates. Several *c*-type cytochromes are associated with the outer membrane of microaerobically or anaerobically grown *S. putrefaciens* MR-1; when reduced, the haems are rapidly reoxidized by the addition of Fe³⁺ [13]. One of these *c*-type cytochromes, the 83 kDa OmcA, has been purified from detergent-solubilized outer-membrane fractions [13]. The sequence of the *omcA* gene has also been determined: it indicates that the translated protein contains ten *c*-haems [14]. Further genetic analysis has revealed *omcA* to be part of a 13 kb gene

Abbreviations used: DCPIP, dichlorophenol-indophenol; Fcc₃, soluble flavocytochrome *c*₃ fumarate reductase; Ifc₃, iron-induced flavocytochrome *c*₃; MV, Methyl Viologen; ORF, open reading frame; PGE, pyrolytic graphite edge.

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cluster, *mtrDEF-omcA-mtrCAB*, that putatively encodes four other decahaem *c*-type cytochromes [15]. Two outer-membrane β -barrel proteins, MtrE and MtrB, are also predicted to be encoded by this nucleotide sequence; transposon insertion into *mtrB* results in *S. putrefaciens* MR-1 becoming unable to respire Fe^{3+} [15].

The periplasmic compartment of *Shewanella frigidimarina* (formerly *S. putrefaciens*) NCIMB400 [16] grown in oxygen-limited or anaerobic culture also accommodates a number of *c*-type cytochromes, with low-potential haems predominating [17]. The two major such proteins have been identified as a 63.8 kDa tetrahaem flavocytochrome c_3 fumarate reductase (Fcc₃) and an 11.8 kDa tetrahaem cytochrome c_3 of unknown physiological function [18]. The presence of equivalent proteins has moreover been demonstrated in *S. putrefaciens* MR-1 [19,20]. These soluble cytochromes of *Shewanella* species might be envisaged to have a role in Fe^{3+} reduction, either in the transfer of electrons from the cytoplasmic membrane to the outer membrane or in the direct reduction of any soluble Fe^{3+} species that might gain access to the periplasm. The Fe^{3+} -respiring micro-organism *Desulfuromonas acetoxidans* also features soluble low-potential *c*-type cytochromes that are purported to be involved in electron transport to the cation [21–23], whereas the Fe^{3+} reductase activity of *Desulfovibrio vulgaris* has been attributed to a periplasmic cytochrome c_3 [24]. Both outer-membrane and periplasmic *c*-type cytochromes have been suggested to be responsible for Fe^{3+} reduction by *Geobacter sulfurreducens* [25,26].

Here we report the purification from *S. frigidimarina* NCIMB400 of a 63.9 kDa periplasmic tetrahaem flavocytochrome c_3 , designated Ifc₃, that is found in cells cultured by the anaerobic respiration of soluble Fe^{3+} complexes with citrate or pyrophosphate ligands. The expression of Ifc₃ does not occur in cells grown either microaerobically or anaerobically with an extensive range of alternative soluble terminal electron acceptors. Ifc₃ is therefore the first protein from any bacterium to be identified as being synthesized only when a metal cation is being reduced to conserve energy for growth. We also report the disruption of the chromosomal *ifcA* gene that encodes Ifc₃ in *S. frigidimarina* NCIMB400, and detail the increased expression during growth on Fe^{3+} of other *c*-type cytochromes for the Ifc₃-deficient mutant strain in comparison with the parent strain.

EXPERIMENTAL

Bacterial strains and cell growth

The strains of *S. frigidimarina* used in this study are listed in Table 1. Growth of the bacterium was performed at 25 °C in the basal medium of Luria–Bertani (g/l: tryptone 10, yeast extract 5, NaCl 10) adjusted to pH 7.5 with NaOH and supplemented with D,L-lactate (50 mM). Kanamycin and/or chloramphenicol (both at 50 µg/ml) were employed where appropriate. Microaerobic growth was achieved by shaking 100 ml cultures in 250 ml conical flasks at 200 rev./min; cells were harvested during late exponential phase. For anaerobic growth with soluble Fe^{3+} as the sole terminal electron acceptor present, the cation was supplied as ferric citrate (5–50 mM) or ferric pyrophosphate (5–20 mM). The alternative soluble respiratory substrates fumarate, nitrate (NO_3^-), nitrite (NO_2^-), trimethylamine-*N*-oxide, tetrathionate ($\text{S}_4\text{O}_6^{2-}$) and thiosulphate ($\text{S}_2\text{O}_3^{2-}$) were used at 25 mM. Anaerobic growth was achieved in completely filled 500 ml bottles, with 5 ml of microaerobically grown cells being employed as the inoculum. Cells obtained by using soluble Fe^{3+} were harvested when reduction of the cation was judged to be complete by a ferrozine-based assay for Fe^{2+} formation [7].

Table 1 Bacterial strains, plasmids and oligonucleotide primers used in this work

Strain/plasmid/ oligonucleotide primer	Description	Reference/source
Strains		
<i>S. frigidimarina</i> NCIMB400	Wild-type <i>S. frigidimarina</i>	National Collections of Industrial and Marine Bacteria, Aberdeen, U.K. Laboratory stock
NCIMB400R	Spontaneous Rif ^r strain	[45]
EG301	NCIMB400R Δ <i>ifcA</i> :: <i>ahp</i> Km ^r	
PSD1	NCIMB400R <i>ifcA</i> :: Ω Cm ^r	
PSD2	EG301 <i>ifcA</i> :: Ω Cm ^r	
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>rec A1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Gibco-BRL
S17-1	<i>thi pro hsdR hdsM⁺ recA</i> , chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7) <i>Strep^r</i>	[49]
Plasmids		
pBluescript II KS +	Cloning vector; Amp ^r	Stratagene
pLitmus 28	Cloning vector; Amp ^r	New England BioLabs [37]
pJQ200KS	Mobilizable suicide vector; Gm ^r	
pH45 Ω Cm	Cloning vector containing Ω Cm; Amp ^r	[50]
pPSD101	1.6 kb <i>EcoRI</i> – <i>HindIII</i> PCR fragment cloned into pBluescript II KS +	This study
pPSD102	808 bp <i>Bam</i> HI– <i>HindIII</i> PCR fragment cloned into pLitmus 28	This study
pPSD103	794 bp <i>EcoRI</i> – <i>Bam</i> HI PCR fragment cloned into pPSD102	This study
pPSD104	3.5 kb <i>Bam</i> HI DNA fragment containing Ω Cm cloned into pPSD103	This study
pPSD105	5 kb <i>Bgl</i> II– <i>Sac</i> I DNA fragment containing Ω Cm-disrupted partial <i>ifcA</i> cloned into pJQ200KS	This study
pPSD106	8 kb <i>Sac</i> I DNA fragment containing Ω Cm-disrupted <i>ifcA</i> and surrounding sequence cloned into pBluescript II KS +	This study
pPSD107	1.5 kb <i>Bam</i> HI DNA fragment containing C-terminal half of <i>ifcA</i> and downstream sequence cloned into pBluescript II KS +	This study
pPSD108	2.8 kb <i>Bam</i> HI– <i>Sac</i> I DNA fragment containing N-terminal half of <i>ifcA</i> and upstream sequence cloned into pBluescript II KS +	This study
Oligonucleotides		
PSD10F	5'-CGT GAA TTC AAR ACN CCN GAY ATG GG-3'	This study
PSD20R	5'-CGC AAG CTT RCC RTG HAC RCC RCC DGT HAC YTC RCC HGC-3'	This study
PSD30R	5'-CGT GGA TCC ATA CCC TGT TCT TTG-3'	This study
PSD40F	5'-GCT GGA TCC ACG ACT AAA CTC TCG-3'	This study

Cell fractionation

S. frigidimarina cells were harvested by centrifugation at 15 900 *g* and 4 °C for 15 min. Periplasmic fractions were prepared by suspending the cells in 5 or 1 ml of 100 mM Tris/HCl (pH 8)/500 mM sucrose/3 mM Na₂EDTA per 100 ml of original microaerobic or anaerobic culture respectively, and incubating them in the presence of 2 mg/ml lysozyme for 20 min at 37 °C. Spheroplasts were removed by centrifugation at 75 600 *g* and

4 °C for 30 min. To prepare membrane fractions, cells were suspended in 5 ml or 1 ml of 20 mM NaHepes, pH 7.5, per 50 ml of original microaerobic or anaerobic culture respectively, then passed twice through a chilled French pressure cell operated at 1000 lb/in² (6.9 MPa), and stirred at 4 °C for 10 min with a few crystals of DNase I. Membranes were pelleted by ultracentrifugation at 208 000 *g* and 4 °C for 1 h; they were then washed with 20 mM NaHepes, pH 7.5, and the ultracentrifugation was repeated.

Protein purification

The haemoprotein observed only in periplasms of Fe³⁺-respiring cells, and later designated Ifc₃ on the basis of biochemical and genetic characterizations, was routinely purified from a total volume of 50 litres of *S. frigidimarina* EG301 batch cell cultures grown anaerobically on 50 mM ferric citrate. All subsequent chromatographic, dialysis and concentration steps were performed at 4 °C. The periplasmic fraction was loaded on a DEAE-Sepharose CL-6B (Pharmacia Biotech) anion-exchange column (40 cm × 2.5 cm) previously equilibrated with 20 mM Tris/HCl (pH 8)/50 mM NaCl. After washing with 2 column vol. of equilibration buffer, a linear gradient of 50–300 mM NaCl was applied over 4 column vol. Ifc₃ was eluted at approx. 190 mM NaCl. All fractions containing Ifc₃ (as indicated by SDS/PAGE) were pooled and (NH₄)₂SO₄ was added to 50 % satn. After the removal of precipitated proteins by centrifugation at 75 600 *g* for 10 min, the resulting solution was loaded on a Phenyl-Sepharose CL-4B (Pharmacia Biotech) hydrophobic interaction column (40 cm × 2.5 cm) previously equilibrated with 20 mM NaHepes (pH 7.5)/2.2 M (NH₄)₂SO₄. Washing with one column vol. of equilibration buffer was followed by the application of a linear gradient of 2.2–0.22 M (NH₄)₂SO₄ in 20 mM NaHepes, pH 7.5, over 4 column vol. Complete elution of Ifc₃ was accomplished by washing with 2 column vol. of 20 mM NaHepes (pH 7.5)/0.22 M (NH₄)₂SO₄. All fractions containing Ifc₃ were pooled and dialysed (3 kDa cut-off membrane) against 20 mM Tris/HCl (pH 8)/50 mM NaCl. The resulting solution was then applied to a DEAE-Sepharose CL-6B anion-exchange column (40 cm × 2.5 cm), which was developed as described above. Fractions containing only Ifc₃ were combined, dialysed against 20 mM NaHepes (pH 7.5)/100 mM NaCl, then concentrated by ultrafiltration (3 kDa cut-off membrane; Amicon) to give a protein concentration of approx. 10 mg/ml.

SDS/PAGE

Analyses of periplasmic extracts, membranes and fractions from column chromatography were routinely performed by SDS/PAGE with a Hoefer Mini-Gel System (10 cm × 8 cm). Slab gels of 10 % or 15 % (w/v) polyacrylamide were employed for the resolution of proteins, with samples being loaded via a stacking gel of 5 % (w/v) polyacrylamide. The samples were prepared for electrophoresis by incubation with 3 M urea/90 mM SDS at room temperature for 1 h. Gels were stained with Coomassie Brilliant Blue R-250. The presence of *c*-type cytochromes was tested for by haem-linked peroxidase staining [27].

Protein sequencing

For N-terminal sequence determination, a 4 μM solution of Ifc₃ was boiled with 700 mM 2-mercaptoethanol/35 mM SDS. The protein was resolved by SDS/PAGE [10 % (w/v) gel] and subsequently electroblotted on PVDF membrane (0.2 μm; Bio-

Rad). For cleavage of Met-Xaa bonds, a solution of Ifc₃ was treated with CNBr by using the protocol of Smith [28]. The resulting peptides were resolved by SDS/PAGE [10–20 % (w/v) linear gradient gel] and electroblotted. Sequence cycles were acquired on an Applied Biosystems Procise Sequencer by Dr. M. Naldrett (John Innes Centre, Norwich, U.K.).

Protein quantification and haem and flavin analyses

Protein concentrations were determined by the bicinchoninic acid method by using the protocol supplied by Sigma, with 0.25 mg/ml BSA as standard. For quantification of the *c*-haems of Ifc₃, conversion into pyridine derivatives was achieved by incubating protein (3 μM) with pyridine (2.1 M) and NaOH (75 mM) in water at room temperature for 15 min. Sodium dithionite and potassium ferricyanide were added to separate aliquots of the resulting solution such that the final concentrations of protein, reductant and oxidant were 2.5 μM, 1.5 mM and 750 μM respectively. Haem content was subsequently determined by using a difference molar absorption coefficient of 19.1 mM⁻¹·cm⁻¹ at 550 nm for the pyridine ferrohaemochrome minus the pyridine ferrihaemochrome spectrum [29]. Flavin analysis was performed by using a modification of a method described previously [30]. A 700 μl sample of 25 μM Ifc₃ was applied to a Pharmacia Biotech PD-10 column (Sephadex G-25; 1 cm × 10 cm) pre-equilibrated with 1.2 M formic acid. Fractions of 0.5 ml were collected on washing the column with equilibration buffer; those containing flavin (as judged by absorption spectra) were combined and diluted to give 10 ml of a solution containing 100 mM K₂HPO₄, pH 7. Flavin content was subsequently determined by using a molar absorption coefficient of 11.3 mM⁻¹·cm⁻¹ at 450 nm for FAD in this buffer [30]. Both absorption spectroscopy and ascending paper chromatography [30] with commercial standards were employed to confirm the source of flavin to be FAD and not FMN.

Spectroscopy

Electronic absorption spectra were acquired on an SLM Aminco DW-2000 UV/visible spectrophotometer. EPR spectra were recorded on a Bruker Spectrospin ER-200D X-band spectrometer equipped with an Oxford Instruments ESR-9 liquid-helium-flow cryostat.

Redox titrations

Mediated redox potentiometry was based on the methodology of Dutton [31]. Titration of 17 μM oxidized Ifc₃ with dithionite was performed under an argon atmosphere at 15 °C in 100 mM NaHepes (pH 7.5)/100 mM NaCl, with wavelength scans of 500–600 nm being acquired after each addition of reductant. A saturated solution of quinhydrone (hydroquinone/benzoquinone 1:1 complex; *E*⁰ = +295 mV) was used for standardization of the Pt-Ag/AgCl combination microelectrode. The mediators employed were diaminodurene (*E*⁰ = +250 mV), phenazine methosulphate (+80 mV), phenazine ethosulphate (+55 mV), juglone (+30 mV), duroquinone (+5 mV), menadione (–80 mV), anthraquinone 2,6-disulphonate (–185 mV), anthraquinone 2-sulphonate (–225 mV) and Benzyl Viologen (–360 mV) (all at 10 μM). The reduction potentials reported for the haems of Ifc₃ are referenced to the standard hydrogen electrode. To obtain samples of Ifc₃ poised at different potentials for EPR spectroscopy, the above methodology was repeated with a 40 μM solution of protein. Aliquots were transferred periodically to anaerobic EPR tubes and immediately frozen in liquid N₂.

Electrochemistry

Cyclic voltammetry was performed with an Autolab electroanalyser (Eco Chemie, Utrecht, Netherlands) with the analogue scan generator controlled by GPES software. The electrochemical cell consisted of a pyrolytic graphite edge (PGE) working electrode, Pt foil counter electrode and reference standard calomel electrode in a Luggin side-arm filled with 20 mM NaHepes (pH 7.5)/100 mM NaCl. This was thermostatically controlled and housed in an anaerobic chamber (Belle Technology, Portesham, Dorset, U.K.) with an O₂ content of less than 2 p.p.m. Immediately before use, the PGE electrode was polished with Al₂O₃ slurry, sonicated, rinsed with water and dried. Potentials are reported with reference to the standard hydrogen electrode by the addition of 241 mV to the value obtained with the standard calomel electrode at 25 °C [32].

Enzymic activity assays

All assays were conducted at 20 °C under anaerobic conditions and with constant stirring. The Fe³⁺ reductase activity of harvested intact cells of *S. frigidimarina* NCIMB400 was quantified by monitoring the disappearance of the soluble complex Fe³⁺(3-hydroxy-2-methyl-4-pyrone)₃ at 450 nm. This assay has previously been described in detail [33]. The fumarate reductase activity of periplasmic fractions and purified Ifc₃ was quantified spectrophotometrically with the redox dye Methyl Viologen (MV) (*E*^{0'} = 445 mV) [34]. Assays were performed with 3 nM Ifc₃ and 1 mM MV buffered with 20 mM NaHepes (pH 7.5)/100 mM NaCl. The MV was reduced with dithionite to give an absorbance of approx. 2 at 600 nm; the disappearance of this species was monitored with regard to time after the addition of 10 mM fumarate. Alternative electron acceptors used in this assay system were trimethylamine-*N*-oxide, tetrathionate, thiosulphate and nitrate. Benzyl Viologen was employed in place of MV for assays with nitrite. The succinate dehydrogenase activity of Ifc₃ was determined spectrophotometrically by monitoring the coupled reduction of dichlorophenol-indophenol (DCPIP) (*E*^{0'} = +220 mV) at 600 nm [35]. The assay mixture contained 300 nM Ifc₃, 2 mM phenazine methosulphate and 25 µM DCPIP buffered with 20 mM NaHepes (pH 7.5)/100 mM NaCl; the reaction was initiated by the addition of 10 mM succinate.

Stopped-flow kinetics

The reaction of dithionite-reduced Ifc₃ (5 µM) with Fe³⁺-EDTA (150 µM) in 100 mM NaHepes (pH 7.5)/100 mM NaCl was monitored in an Applied Photophysics Bio-Sequential DX.17MV stopped-flow spectrophotometer with a 1 cm path-length cell. Detection at multiple wavelengths was achieved with an Applied Photophysics photodiode array accessory. Both enzyme and substrate solutions were prepared in an anaerobic cabinet with deoxygenated buffer containing glucose (2 mM), glucose oxidase (20 units/ml) and catalase (300 units/ml). Ifc₃ was titrated to full reduction with dithionite by using a Pharmacia LKB Ultraspec III UV/visible spectrophotometer within the cabinet.

Molecular mass determination

A molecular mass measurement for Ifc₃ was made with a Ciphergen Biosystems surface-enhanced laser desorption/ionization ProteinChip Molecular Recognition System, MRS-1. Chicken conalbumin (77490 Da) and horseradish peroxidase (43240 Da) were employed for internal calibration.

General molecular biology

The bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Table 1. *Escherichia coli* was grown in Luria–Bertani medium at 37 °C. Antibiotics were added where appropriate to give the following concentrations (µg/ml): ampicillin, 100; chloramphenicol, 50; gentamicin, 10; kanamycin, 50; rifampicin, 10; streptomycin, 50. Southern blots on positively charged nylon membrane (Boehringer Mannheim) were hybridized at 68 °C. A DIG High Prime Kit (Boehringer Mannheim) was employed for the labelling of probes and colour-based detection. Nucleotide sequencing was performed by MWG-Biotech UK.

Construction of ifc₃-deficient mutant strains

Degenerate oligonucleotide primers PSD10F and PSD20R respectively were designed to complement the codons for the N-terminal amino acid sequence of Ifc₃ (with an additional *Eco*RI cleavage site) and amino acid residues 531–540 of Fcc₃ (with an additional *Hind*III cleavage site). The internal sequence of A⁵³¹GEVTGGVHG⁵⁴⁰ for Fcc₃ is conserved in the FccA protein from *Wolinella succinogenes* [36] and is most probably essential for flavin binding. A 1.6 kb product obtained from PCR with *S. frigidimarina* NCIMB400 genomic DNA as template was cloned into pBluescript II KS+ and transformed into *E. coli* DH5α. The resultant plasmid, pPSD101, was sequenced to confirm that the product from PCR corresponded to a portion of *ifcA*, i.e. the gene encoding Ifc₃. Specific oligonucleotide primers PSD30R and PSD40F were then designed with *Bam*HI cleavage sites replacing bases 795–800 (i.e. TGA TAC) of the partial *ifcA* sequence. These were used for PCRs in combination with the previously described degenerate primers (i.e. PSD10F with PSD30R, and PSD40F with PSD20R) and pPSD101 as template, the two products then being sequentially cloned into pLitmus 28 to give pPSD103. An Ω-cassette encoding chloramphenicol resistance, excised from pHP45 on a *Bam*HI fragment, was next ligated into pPSD103 that had been cut with the same enzyme. From the resultant pPSD104 the partial *ifcA* gene with inserted Ω-cassette was excised on a *Bgl*II–*Sac*I fragment and ligated into the suicide vector pJQ200KS cut with *Bam*HI and *Sac*I. The final plasmid, pPSD105, was transformed into *E. coli* S17-1 and used in subsequent matings with *S. frigidimarina* strains NCIMB400R and EG301 at 25 °C. Selection for double-recombinant mutants was achieved on agar plates containing 50 mg/ml sucrose [37]. The mutants of *S. frigidimarina* NCIMB400 in *ifcA*, and in both *ifcA* and *fccA*, were designated strains PSD1 and PSD2 respectively.

Gene cloning

Genomic DNA from the *ifcA* mutant *S. frigidimarina* PSD1 was digested with *Sac*I and resolved by fragment size on a 10–60% (w/v) sucrose gradient by ultracentrifugation in a Beckman SW28 rotor operating at 25000 rev./min (141000 *g*) and 13 °C for 18 h. Southern blots of fractions obtained were hybridized with probes prepared from both the chloramphenicol-resistance cassette and the partial *ifcA* gene obtained by PCR. Fractions revealed to contain the appropriate DNA were shotgun-cloned into pBluescript II KS+ and transformed into *E. coli* DH5α; selection was achieved on plates containing chloramphenicol. The antibiotic-resistance cassette was removed from the resultant plasmid on a *Bam*HI fragment; religation/cloning was employed to give the final plasmids pPSD107 and pPSD108. These were

subsequently sequenced to give complete data for disrupted *ifcA* plus surrounding DNA.

RESULTS

Expression and purification of Ifc₃

Anaerobic growth on soluble Fe³⁺ was achieved for both *S. frigidimarina* NCIMB400 and the Fcc₃-deficient mutant strain EG301. Complete consumption of respiratory substrate within 48 h was noted for all concentrations of ferric citrate and ferric pyrophosphate supplied. A growth yield of 2.5 g of total cell protein synthesized per mol of Fe³⁺ reduced was obtained by using *S. frigidimarina* NCIMB400 and 50 mM ferric citrate, in broad agreement with published findings on *S. putrefaciens* MR-1 [7]. By using a previously established method for assaying the reduction of soluble Fe³⁺ species by harvested intact cells [33], and with lactate and formate both present as electron donors, *S. frigidimarina* NCIMB400 grown anaerobically with 50 mM Fe³⁺ citrate gave a specific activity of 1.3 μ mol of Fe³⁺(3-hydroxy-2-methyl-4-pyrone)₃ reduced/min per mg of cell protein. Corresponding values of 0.33 and 0.28 respectively were obtained for cells grown anaerobically with 25 mM fumarate and microaerobically. These results suggested that an induction of Fe³⁺ reductase activity occurred during anaerobic growth with ferric citrate. In view of the potential roles for periplasmic *c*-type cytochromes in Fe³⁺ reduction, the expression of such soluble proteins was examined in cultures with Fe³⁺ in comparison with cultures with other respiratory substrates. Thus periplasms were prepared from *S. frigidimarina* NCIMB400 and EG301 cells grown anaerobically on 50 mM ferric citrate; they were subjected to SDS/PAGE with the same cell fraction of the parent strain grown both anaerobically with 25 mM fumarate and microaerobically (Figure 1A). For the NCIMB400 cultures, the 63.8 kDa Fcc₃ was identified as one of the major protein bands, running nearly adjacent to a 66.25 kDa marker (Figure 1A). However, growth on soluble Fe³⁺ yielded a band running just below the Fcc₃ that was absent from the periplasmic fractions of NCIMB400 grown either anaerobically on fumarate or microaerobically (Figure 1A). This band was also found in the periplasm of the Fcc₃-deficient mutant EG301 cultured with 50 mM ferric citrate (Figure 1A) and gave a positive haem-linked peroxidase stain (Figure 1B). Further SDS/PAGE analysis demonstrated that an equivalent band was derived from periplasms of both parent and mutant strains grown on all concentrations of ferric citrate and ferric pyrophosphate but not from periplasms of the bacteria grown anaerobically with all other soluble terminal electron acceptors tested (results not shown). Purification to homogeneity of the protein represented by the band was achieved from EG301 with anion-exchange and hydrophobic-interaction resins (Figure 1, lanes 6); yields of 10 mg from 50 litres of culture were typically obtained. The mutant strain was routinely used in preparations because some co-elution with Fcc₃ was noted during the first chromatographic step. Subsequent biochemical and genetic characterizations indicated that the periplasmic protein synthesized during respiration of soluble Fe³⁺ by *S. frigidimarina* NCIMB400 was a tetrahaem flavocytochrome *c*₃, which we have designated Ifc₃ (iron-induced flavocytochrome *c*₃).

UV/visible spectroscopy and haem analysis

The visible spectrum of the reduced haemoprotein Ifc₃ (Figure 2) featured absorption maxima at 552, 523 and 420 nm (α , β and γ bands respectively) that are diagnostic of *c*-type cytochromes. Absorption coefficients calculated from a molecular mass of

Figure 1 SDS/PAGE [10% (w/v) gel] profiles of *S. frigidimarina* periplasmic fractions, membrane fractions and purified protein

(A) Coomassie Blue stain; (B) haem-linked peroxidase stain. Lanes 1, molecular mass standards (10 μ g) (hen's-egg ovotransferrin, 76–78 kDa; BSA, 66.25 kDa; hen's-egg ovalbumin, 42.7 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa); lanes 2, periplasm from microaerobic culture of *S. frigidimarina* NCIMB400 (40 μ g); lanes 3, periplasm from NCIMB400 grown anaerobically with 25 mM fumarate (40 μ g); lanes 4, periplasm from NCIMB400 grown anaerobically with 50 mM ferric citrate (80 μ g); lanes 5, periplasm from *S. frigidimarina* EG301 grown anaerobically with 50 mM ferric citrate (80 μ g); lanes 6, a purified sample of the protein synthesized during the respiration of soluble Fe³⁺ and designated Ifc₃ (3 μ g); lanes 7, periplasm from *S. frigidimarina* PSD1 grown anaerobically with 50 mM ferric citrate (80 μ g); lanes 8, periplasm from *S. frigidimarina* PSD2 grown anaerobically with 50 mM ferric citrate (80 μ g); lane 9, membranes from NCIMB400 grown anaerobically with 50 mM ferric citrate (60 μ g); lane 10, membranes from PSD1 grown anaerobically with 50 mM ferric citrate (60 μ g).

64 kDa for Ifc₃ are 110 (α), 60 (β) and 620 (γ) mM⁻¹·cm⁻¹. The UV/visible spectrum of oxidized Ifc₃ (Figure 2) gave an *A*₄₁₀-to-*A*₂₈₀ ratio of 4.5. Assuming a molecular mass of 60 kDa for the Ifc₃ apoprotein, the pyridine haemochrome spectra

Figure 2 UV/visible absorption spectra of Ifc₃

Purified protein was diluted with 20 mM NaHepes (pH 7.5)/100 mM NaCl to give a concentration of 0.11 mg/ml. Reduction was achieved by the addition of excess solid dithionite. Solid line, oxidized protein; broken line, reduced protein.

Figure 3 Spectrophotometric-mediated reductive titration of Ifc₃

The solid line corresponds to a best fit between the data and four Nernst curves, assuming equal spectral contributions from each haem. These curves are centred at -73, -141, -174 and -259 mV.

indicated that 3.7 mol of *c*-haem was present per mol of polypeptide, which suggested that Ifc₃ is a tetrahaem species.

Spectrophotometric redox titration

Values obtained for $A_{552} - A_{560}$ (i.e. the absorbance at the $\alpha_{\max}$ wavelength for reduced *c*-haem minus the absorbance at an isosbestic point) in the spectrophotometric-mediated reductive titration of Ifc₃ with dithionite were plotted as a function of E (Figure 3). A theoretical curve comprising four Nernstian components of equal amplitude centred at -73, -141, -174 and -259 mV gave a best fit to the data (Figure 3), i.e. low midpoint potentials that are characteristic of bis-His-ligated *c*₃-haems. Reversibility of the titration was confirmed by using ferricyanide, which yielded midpoint potential values within 5% of those reported.

Figure 4 EPR spectrum of Ifc₃

Experimental conditions: temperature 10 K; modulation amplitude 1 mT; microwave power 2 mW; microwave frequency 9.4 GHz. Adventitiously bound Fe³⁺ was removed from the Ifc₃ sample by dialysis against 20 mM NaHepes (pH 7.5)/100 mM NaCl/10 mM EDTA.

EPR spectroscopy

The EPR spectrum of fully oxidized Ifc₃ (Figure 4) was found to contain a dominant rhombic trio ($g_z = 2.88$, $g_y = 2.24$, $g_x = 1.55$) arising from low-spin ($S = \frac{1}{2}$) haem. For a second such set of haem g values, only a g_z feature at 3.17 was apparent (Figure 4), suggesting the presence of highly anisotropic low-spin haem. Spin quantification at the two g_z values revealed signals of equal intensities, indicating that each represent two of the four haems present in the protein. From the spectra obtained with EPR samples of Ifc₃ poised in different redox states, the peak heights at the dominant g_z of 2.88 were assumed to be directly proportional to the fraction of the haem represented by this signal that was oxidized, and were therefore plotted as a function of E (Figure 5). The resultant data were found to fit quite reasonably to a Nernstian curve invoking the midpoint potentials of -73 and -259 obtained in the spectrophotometric redox titration of the protein (Figure 5), but did not fit to equivalent curves constructed with any other pairing from the four previously deduced haem potentials as the set parameters. A similar plot with the g_z of 3.17 could not be confidently obtained owing to a peak broadening observed with the lowering of E . However, titration of the signal from fully oxidized to fully reduced occurred over the approximate range -100 to -200 mV.

Flavin analysis and fumarate reductase activity

Spectrophotometric quantification of the acid-labile flavin of Ifc₃ yielded a ratio of 0.96 mol of FAD per mol of polypeptide with the molecular mass of the apoprotein taken as 60 kDa. The use of Ifc₃ in a standard assay with the redox dye MV yielded a specific activity of 419 μ mol of fumarate reduced/min per mg; no significant reduction of alternative substrates was apparent. For comparison, the specific activity of Fcc₃, the physiological fumarate reductase of *S. frigidimarina* NCIMB400, was 197 μ mol

Figure 5 EPR redox titration of Ifc₃ at $g_z = 2.88$

The solid line corresponds to a fit between the data and two Nernst curves of equal contribution centred at -73 and -259 mV.

of fumarate reduced/min per mg. Negligable succinate dehydrogenase activity was detected for Ifc₃ in assays with DCPIP, indicating that fumarate reduction by the protein was unidirectional.

Electrochemistry

Cyclic voltammetry of Ifc₃ produced complex waveforms with an average position of -191 ± 15 mV (Figure 6A). The dominant features of the reductive and oxidative waves were of an equal magnitude that varied linearly with respect to the square root of the scan rate at 20 mV/s or less; this is the expected behaviour of a redox protein in solution undergoing reversible electron transfer with an electrode. The large peak separation of 150 ± 20 mV obtained for Ifc₃, as well as the shoulders present on the main features, suggest that a series of overlapping waves are observed in a range encompassing the low midpoint potentials of the c_3 -haems of the protein. Moreover, the absence of additional features in the Ifc₃ voltammograms over the range -700 to $+500$ mV indicates that the reduction potential of the non-covalently bound FAD of the protein lies close to the haem values. Consistent with this finding is the observation that the addition of fumarate to Ifc₃ in the electrochemical cell produced an alteration in the voltammetric response to that typical of catalytic reduction (Figure 6B); the potential at which the rate of current change was a maximum was -175 ± 15 mV. This value is considerably lower than the formal fumarate/succinate couple of $+33$ mV. Similar catalytic oxidation of succinate by Ifc₃ was not observed (Figure 6B), complementing the findings from conventional assays that the fumarate reductase activity of the protein is unidirectional.

Stopped-flow kinetics

The reaction between $5 \mu\text{M}$ reduced Ifc₃ (i.e. $30 \mu\text{M}$ electrons) and $150 \mu\text{M}$ Fe³⁺-EDTA was monitored in a stopped-flow spectrophotometer (results not shown). The kinetics of haem oxidation observed at 552 nm (i.e. A_{max}) were monophasic and reaction was essentially complete within 30 ms ($k_{\text{obs}} = 120 \text{ s}^{-1}$). Second-order dependence on substrate concentration was indicated by experiments in which the molar excess of Fe³⁺-EDTA over protein was altered. Results obtained with either Fcc₃ or the 11.8 kDa tetrahaem cytochrome c_3 from *S. frigidimarina*

Figure 6 Cyclic voltammograms of Ifc₃

Conditions: (A) $80 \mu\text{M}$ protein, 4°C ; (B) 500 nM protein plus 2 mM fumarate (solid line) or 6 mM succinate (broken line), 25°C . The scan rate was 5 mV/s at pH 7.5. The second voltammetric sweeps obtained at a freshly polished PGE electrode are presented.

NCIMB400 in place of Ifc₃ were not significantly different from the above.

Analysis of the *ifcA* gene locus

An open reading frame (ORF) of 1764 bp corresponding to *ifcA* was located on a 4275 bp *SacI* fragment of *S. frigidimarina* NCIMB400 chromosomal DNA. The gene encodes the 566 amino acid residues of the mature protein Ifc₃ and a putative signal peptide of 22 residues, with the cleavage site being evident from the N-terminal protein sequence data of K¹TPDMGSFHA¹⁰. Assignment of the ORF as *ifcA* was supported by identification in the translated protein of the internal peptide sequences derived from digests of Ifc₃ with CNBr, namely N¹⁷³AVGTKQQT¹⁸² and K¹⁹⁸GGRQQNDIKLVT-IL²¹². The Ifc₃ apoprotein is predicted to have a molecular mass of 60679 Da and a pI of 7.32. The incorporation of four c -haems and non-covalently bound FAD increased the molecular mass to 63928 Da, which lies within the range 63985 Da with an error of $\pm 0.1\%$ determined by surface-enhanced laser desorption/ionization MS.

Database searches revealed the closest relative of Ifc₃ to be Fcc₃, the physiological fumarate reductase from *S. frigidimarina* NCIMB400 [38], exhibiting 43% identity and 53% similarity.

1	50
Ifc3	MKLYLVSAM ALVVLSSGTA MA...KTPDM GSFHADMGSC QSCHA...KPI
Fcc3	MKQMLAVCI ATLMGTAGLM GTAVA...ADNL AEFHVQNEC DSCHTPD...GE
51	100
Ifc3	KVTDSETHEN AQCKSCHGEY AELA...NDKL QFDPHNSHL GDINCTSCHK
Fcc3	LSNDSLTYEN TQCVSCHGTL AEVAETTKHE HYNASHHFF GEVACTSCHS
101	120
Ifc3	GHEEPKFYCN ECHSFDIKPM
Fcc3	AHEKSMVYCD SCHSFDNF.M

Figure 7 Amino acid sequences of the cytochrome (N-terminal) domains of Ifc₃ and Fcc₃

The residues that constitute the putative signal peptides are italicized. The four conserved haem-binding motifs CXXCH are underlined, as are the four conserved histidine residues that might provide ligands to give c₃-haems.

Figure 8 *SacI* fragment (4275 bp) of the *S. frigidimarina* NCIMB400 genome containing *ifcA*

Both *ifcA* and *orf1* correspond to completely sequenced genes, whereas *orf2* corresponds to a partly sequenced gene.

Both Ifc₃ and Fcc₃ featured N-terminal cytochrome domains (Figure 7), with four haem-binding motifs of CXXCH, and four histidine residues predicted to act as the sixth ligands to give c₃-haems. The two proteins also each possess a C-terminal flavin domain, into which is incorporated a molecule of non-covalently bound FAD; conserved in Ifc₃ are the Asp¹⁹⁵, His³⁶⁵, Arg³⁸¹ and Arg⁵⁴⁴ residues of Fcc₃ that have been proposed to be involved in the mechanism for fumarate reduction [18]. The putative signal peptides for Ifc₃ and Fcc₃ [38] show little similarity (Figure 7), although both must direct the localization of protein to the periplasmic compartment.

Upstream of the ATG initiation codon for *ifcA* are located a putative Shine–Delgarno sequence of AGGA at bases –12 to –9, a putative –10 box of TAATAT at bases –55 to –50 and the sequence TTGAT_NATCTA at bases –91 to –78. The latter nucleotides agree strongly with the reported consensus of the binding site for oxygen-responsive transcriptional activators of the Fnr family, which positively regulate enzymes of anaerobic respiration [39]. The *fnr* analogue *etrA* has been characterized in *S. putrefaciens* MR-1 [40]; a similar gene would seem likely to be present in *S. frigidimarina* NCIMB400. A potential rho-independent termination signal downstream of the TAG stop codon of *ifcA* is suggested by inverted repeats in the range +35 to +58 and a run of thymines at bases +59 to +66.

Another completely sequenced ORF, designated *orf1*, lies upstream of *ifcA* on the 4275 bp *SacI* fragment; the two genes have opposite polarities (Figure 8). The *orf1* gene encodes a putative mature protein of 324 residues, with a predicted signal peptide of 27 residues. Primary sequence analysis and database searches suggested that Orf1 was most probably a β-barrel protein that is associated with the outer membrane of *S. frigidimarina* NCIMB400. The closest relative of Orf1 was found to be the Omb3 of *Neisseria meningitidis*, an outer-membrane

protein that serves as a slightly cation-selective porin [41]. However, these two proteins exhibit only 20 % identity and 27 % similarity. An incomplete ORF, designated *orf2*, is located at the 5' end of the sequenced 4275 bp *SacI* fragment; this also has a polarity opposite to that of *ifcA* (Figure 8). Database searches suggested that the 142 residues of the partial protein Orf2 were most closely related to a range of transcriptional regulators from the LysR family, which are normally of around 300 residues. Identities and similarities between Orf2 and the N-terminal regions of these proteins were at most 35 % and 50 % respectively [42].

Characterization of the *ifcA* mutant strains

The ability to grow anaerobically on 50 mM ferric citrate was retained in both *S. frigidimarina* PSD1 and PSD2, the *ifcA* and *ifcA/fccA* mutant strains respectively. The supply of respiratory substrate was completely consumed over time scales that were not significantly different from those observed with the parent strain NCIMB400 and the *fccA* mutant EG301. After fractionations of cells from cultures grown on Fe³⁺, both enzymic assays and gel electrophoresis were performed to characterize the mutants. Periplasms of the four strains were found to possess the following relative fumarate reductase activities: NCIMB400, 1.0; EG301, 0.61; PSD1, 0.50; PSD2, 0.0016, i.e. results suggesting the absence of the soluble Ifc₃ protein from strains PSD1 and PSD2. This was confirmed by SDS/PAGE of these periplasms, with no band corresponding to Ifc₃ being developed by Coomassie Blue (Figure 1A) or haem-linked peroxidase stains (Figure 1B). Also apparent from the latter staining technique was an increased synthesis of a 35 kDa periplasmic c-type cytochrome in the strains PSD1 and PSD2 compared with NCIMB400 and EG301 (Figure 1B). This protein has a molecular mass comparable with that of the putative periplasmic decahaem c-type cytochrome MtrA of *S. putrefaciens* MR-1 [15]. Because nucleotide sequencing has demonstrated that *mtrA* is located in the same operon as *mtrC*, which encodes a putative 75 kDa outer-membrane decahaem c-type cytochrome in MR-1, we examined membrane fractions of *S. frigidimarina* NCIMB400 and PSD1 from cultures grown on Fe³⁺ for the presence of a protein equivalent to MtrC. However, haem-linked peroxidase staining revealed the only difference to be the significant expression of a 45 kDa membrane-associated c-type cytochrome in the *ifcA* mutant PSD1 that is scarcely present in the parent strain NCIMB400 (Figure 1B). Similarly, a much higher level of this protein was present in the membrane fraction of the *ifcA/fccA* mutant PSD2 grown on Fe³⁺ than in that of the *fccA* mutant EG301 (results not shown).

DISCUSSION

We have undertaken the first biochemical investigation of the expression of c-type cytochromes in the periplasmic compartment of a bacterium cultured anaerobically with a metal cation serving as the terminal electron acceptor. Previous studies on proteins implicated in bacterial respiratory Fe³⁺ reduction have concentrated on membrane-associated [10,13,14] and periplasmic [20,23,26] c-type cytochromes that are expressed constitutively under anaerobic conditions. However, our work has yielded the first identification and subsequent characterization of a protein that is induced only during the respiration of soluble Fe³⁺. After growth on ferric citrate or ferric pyrophosphate, *S. frigidimarina* NCIMB400 was found to have synthesized a periplasmic tetrahaem flavocytochrome c₃, which we have designated Ifc₃. This protein was not present in periplasm prepared from cells cultured

either anaerobically with other soluble respiratory substrates or microaerobically. Nucleotide sequencing data from upstream of the *ifcA* gene indicate that the anaerobic expression of Ifc₃ is regulated by an Fnr-like protein. However, this must act in synergy with a second transcription factor that is somehow responsive to Fe³⁺ respiration. A similar mode of regulation might operate for other periplasmic *c*-type cytochromes of *S. frigidimarina* NCIMB400, because a number of these were noted to be present at higher levels in cells grown on Fe³⁺ than on alternative respiratory substrates.

Primary sequence analysis revealed the closest relative of Ifc₃ to be Fcc₃, the tetrahaem flavocytochrome *c*₃ of *S. frigidimarina* NCIMB400 that is expressed under microaerobic and all anaerobic growth conditions, and functions as a physiological fumarate reductase. The similarity between Ifc₃ and Fcc₃ is also apparent in the behaviour of redox centres. Regarding the bis-His ligated haems present, midpoint reduction potentials for Fcc₃ have recently been reported as -102, -146, -196 and -238 mV at pH 7.0 [43], whereas comparison of the EPR spectra [44] reveals shifts in *g* values that most probably arise only from differences in the geometry of haem-His planes within the two proteins. The non-covalently bound FAD of Fcc₃ also has a low midpoint reduction potential: a value of -152 mV has been reported at pH 7.0 [43]. As with Fcc₃ [43], Ifc₃ possesses a unidirectional fumarate reductase activity. However, Ifc₃ is not synthesized during the anaerobic respiration of fumarate by *S. frigidimarina* NCIMB400, and the *fccA* mutant EG301 does not grow with fumarate as the sole electron acceptor present [45]. These findings demonstrate that Ifc₃ is not produced by the bacterium in order to reduce fumarate. When cultured with ferric citrate, the *S. frigidimarina* mutant deficient in Ifc₃, strain PSD1, produces both 35 kDa soluble and 45 kDa membrane-associated *c*-type cytochromes at significantly higher levels than does the parent strain. In contrast, the amounts of other haemoproteins synthesized during similar growth of the Fcc₃-deficient strain EG301 are essentially the same as for NCIMB400. These findings, in conjunction with the expression of Ifc₃ only in cells cultured with Fe³⁺, suggest that, whereas Ifc₃ is almost certainly involved in electron transport to the cation, the closely related protein Fcc₃ does not have a significant role in Fe³⁺ respiration. Supporting evidence for the versatility of flavocytochrome *c*₃ in electron transport is provided by the FccA and FccB proteins of *W. succinogenes*, which are related to the flavin and haem domains of Fcc₃ respectively and are postulated to form an soluble oxidoreductase complex [36]. Although induced by growth with fumarate as the terminal electron acceptor, FccA and FccB are not required for respiration of this substrate by the bacterium. Moreover, periplasmic fractions containing these proteins do not reduce fumarate in MV assays.

It is becoming increasingly apparent that *c*-type cytochromes most probably function as Fe³⁺ reductases in *Shewanella* [13,14], *Geobacter* [25,26] and *Desulfuromonas* [23] species. The mechanism of Fe³⁺ reduction would presumably be long-range electron transfer, which might account for the vast array of soluble Fe³⁺ species reduced by *S. putrefaciens* cells [33] and the ability of Fe³⁺-respiring bacteria to grow on soluble complexes of the cation with citrate, pyrophosphate and nitrilotriacetate [46]. The question of whether Ifc₃ functions as a terminal Fe³⁺ reductase in the anaerobic metabolism of *S. frigidimarina* NCIMB400 cannot be answered by biochemical assays alone. The protein rapidly reduces soluble Fe³⁺-EDTA in a stopped-flow kinetic experiment but this finding was expected because of the low midpoint potentials of the *c*₃-haems of Ifc₃ in comparison with the midpoint potential of the Fe³⁺-chelate employed (*E*^{0'} = +114 mV). In view of the presence in *Shewanella* periplasm of

anaerobically induced, low-potential cytochromes *c* that might also reduce Fe³⁺ [17,18,20], it is questionable why a soluble protein such as Ifc₃, which contains similar haems, needs to be synthesized for respiration of the cation. The low-potential non-covalently bound FAD found in Ifc₃ might be envisaged to influence any Fe³⁺ reductase activity of the protein. Flavins can act as single-electron shuttles via the formation of semiquinone radicals. Although spectroscopic evidence for such transient species was not apparent in the stopped-flow kinetic reaction between Ifc₃ and Fe³⁺-EDTA, this might have been precluded by the dominant haem absorbances. Furthermore, no semiquinone was detected during the EPR redox titration of Ifc₃, suggesting a low stability of the radical in the flavoprotein. A role has been postulated for flavins in Fe³⁺ reduction during the assimilation of the metal by bacteria [47], but in this process NAD(P)H serves as an electron donor to the flavin, as opposed to the low-potential *c*₃-haems of Ifc₃. Because *S. frigidimarina* NCIMB400 produces Fcc₃ constitutively under all anaerobic conditions and this protein also contains low-potential FAD, it is again questionable why a closely related flavoprotein such as Ifc₃ should be produced by the bacterium for the purpose of Fe³⁺ respiration.

To be directly reduced by periplasmic proteins such as Ifc₃, it seems most likely that a soluble Fe³⁺ substrate would have to penetrate the outer membrane of *S. frigidimarina* NCIMB400 and that this would occur via passage through porins. However, recent studies on the *mtrCAB* operon of *S. putrefaciens* MR-1 could suggest the failure of periplasmic proteins to act as terminal Fe³⁺ reductases in this bacterium, in that a mutant deficient in the outer-membrane β -barrel protein MtrB did not reduce ferric citrate [15]. MtrB, which is constitutively expressed under anaerobic conditions, has been postulated to contain a metal-binding site that might be of importance in the reduction of Fe³⁺ at the outer membrane [15]. Other possible functions for the protein are as a structural linkage between the 40 kDa periplasmic decahaem MtrA and the 75 kDa outer-membrane decahaem MtrC or as a carrier of electrons between these two cytochromes, with peripheral attachment of MtrA to the periplasmic face of the outer membrane. A respiratory pathway might then be envisaged that involves the passage of electrons from the tetrahaem cytochrome CymA [10], which is anchored to the cytoplasmic membrane, to Fe³⁺ species at the cell exterior, via the periplasmic cytochrome MtrA and the outer-membrane cytochrome MtrC. A 35 kDa cytochrome *c*, i.e. with a molecular mass comparable with that of MtrA, is found in the periplasmic fraction of *S. frigidimarina* NCIMB400 grown on Fe³⁺. We have recently purified this protein and identified the presence of several low-potential *c*₃-haems (M. Ellington, S. J. Field, C. S. Butler, P. S. Dobbin and D. J. Richardson, unpublished work). The syntheses of the 35 kDa soluble cytochrome and also of a membrane-associated 45 kDa cytochrome are markedly increased in the *ifcA* mutant strain PSD1. An operon comparable with *mtrCAB* therefore seems likely to exist in *S. frigidimarina* NCIMB400; moreover, Ifc₃ seems to substitute for the cytochromes encoded by this operon during Fe³⁺ respiration. The low molecular mass for the proposed MtrC analogue compared with the putative 75 kDa protein from *S. putrefaciens* MR-1 might reflect a variation previously noted in both the number and sizes of membrane-associated cytochromes *c* from different *Shewanella* species [14].

The provision of an alternative route for electron transport to Fe³⁺ by Ifc₃, as well as the apparent role for this protein but not the closely related Fcc₃ during growth on Fe³⁺, could be explained by a peripheral attachment of Ifc₃ to the outer membrane. Such an association might allow Ifc₃ to function in a similar manner to that postulated for MtrA, i.e. shuttling electrons from the

periplasm to an outer-membrane Fe^{3+} reductase. Preliminary X-ray diffraction data suggests that Ifc_3 crystallizes as a dimer [48]; it might be envisaged that two molecules of this protein interact in the periplasm to facilitate electron transfer to the outer membrane. In a contrasting model, the positioning of Ifc_3 in close proximity to an outer-membrane porin could perhaps facilitate a direct reduction of soluble Fe^{3+} by this protein that is not achieved by Fcc_3 . The *orf1* gene, found upstream of *ifcA* on the *S. frigidimarina* NCIMB400 genome, encodes a putative 35 kDa outer-membrane protein. However, alignments of Orf1 with the 75 kDa MtrB yielded no significantly similar regions; whereas the closest relation to Orf1 is an outer-membrane porin, the 20% identity between these proteins is not sufficient to suggest that Orf1 functions equivalently. The divergence of and distance between the genes encoding Ifc_3 and Orf1 also indicate that the proteins are transcribed separately; possible interactions with respect to Fe^{3+} respiration are therefore currently unclear. Similarly, an involvement of the putative transcriptional regulator Orf2 cannot be gleaned from our current results.

In summary, we have demonstrated a possible multiplicity of electron transport pathways to Fe^{3+} during respiration of the cation by *S. frigidimarina* NCIMB400. One such pathway seems to involve Ifc_3 , a periplasmic protein that is expressed only in cells grown on Fe^{3+} . We intend to perform further molecular studies on respiratory Fe^{3+} reduction by *S. frigidimarina* NCIMB400, addressing in particular how the transcription of Ifc_3 is activated during this process.

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