Identification and characterization of a novel cytochrome c_3 from *Shewanella frigidimarina* that is involved in Fe(III) respiration

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Shewanella frigidimarina NCIMB400 is a non-fermenting, facultative anaerobe from the gamma group of proteobacteria. When grown anaerobically this organism produces a wide variety of periplasmic *c*-type cytochromes, mostly of unknown function. We have purified a small, acidic, low-potential tetrahaem cytochrome with similarities to the cytochromes c_3 from sulphate-reducing bacteria. The N-terminal sequence was used to design PCR primers and the *cctA* gene encoding cytochrome c_3 was isolated and sequenced. The EPR spectrum of purified cytochrome c_3 indicates that all four haem irons are ligated by two histidine residues, a conclusion supported by the presence of eight histidine residues in the polypeptide sequence, each of which is conserved in a related cytochrome c_3 and in the cytochrome domains of flavocytochromes c_3. All four haems exhibit low midpoint redox potentials that range from -207 to -58 mV at pH 7; these values are not significantly influenced by pH changes. *Shewanella* cytochrome c_3 consists of a mere 86 amino acid residues with a predicted molecular mass of 11780 Da, including the four attached haem groups. This corresponds closely to the value of 11778 Da estimated by electrospray MS. To examine the function of this novel cytochrome c_3 we constructed a null mutant by gene disruption. *S. frigidimarina* lacking cytochrome c_3 grows well aerobically and its growth rate under anaerobiosis with a variety of electron acceptors is indistinguishable from that of the wild-type parent strain, except that respiration with Fe(III) as sole acceptor is severely, although not completely, impaired.

Key words: electron transfer, iron respiration, redox potentials.

INTRODUCTION

Shewanella spp. are widespread Gram-negative bacteria that have been isolated from many different habitats. This adaptability is supported by a flexible metabolic capability, particularly in the choice of respiratory electron acceptors [1,2]. Of particular note is the unusual capacity of *Shewanella* to use insoluble Fe(III) and Mn(IV) compounds as terminal oxidants, which raises important questions about how electrons are transferred to the extracellular medium. The presence of cytochromes in the outer membrane in these bacteria might provide a channel for electron transfer from the periplasm [3]. *Shewanella frigidimarina* NCIMB400 is a facultative aerobe that under anaerobic conditions produces at least nine different cytochromes in the periplasm [4]. One of these is a 64 kDa soluble flavocytochrome c_3 that functions as a respiratory fumarate reductase [5–7]. Spectroscopic analysis has shown that all four haem groups in the cytochrome domain of flavocytochrome c_3 are bis-histidine ligated and titrate at very low redox potentials [8,9]. The gene encoding this enzyme has been isolated and sequenced; its crystal structure has been determined at 1.8 Å (1Å = 0.1 nm) resolution [5,10,11]. In contrast, the structures and functions of other periplasmic components of the anaerobic respiratory chain are poorly understood.

Cytochromes *c* are common components of electron transfer pathways and, particularly in bacterial systems, are remarkably diverse in their molecular properties. Ambler [12] has classified these proteins according to sequence and structural relationships, with multihaem *c*-type cytochromes being grouped together as class III. The best characterized of these proteins are the cytochromes c_3 from obligately anaerobic, sulphate-reducing bacteria [13,14]. These are generally small, low-potential, tetrahaem proteins found in the periplasm that act as electron acceptors from hydrogenase. Related proteins with 8 and 16 haems have been found in addition to the tetrahaem cytochrome c_3 in *Desulfovibrio vulgaris*; these seem, on the basis of their amino acid sequences, to be composed of tandem tetrahaem domains [15,16]. Here we describe the purification of a low-potential, tetrahaem *c*-type cytochrome from *S. frigidimarina* and its characterization by spectroscopy, electrochemistry and sequence analysis. We have designated this protein as a cytochrome c_3 because it shares several properties with the *Desulfovibrio* proteins but the sequences are not closely related.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids

Escherichia coli JM109 was used for general recombinant DNA procedures and DH5α for manipulations involving the shuttle vector pJQ200KS. Growth of *S. frigidimarina* NCIMB400 was as described by Pealing et al. [5] except that Luria–Bertani broth or agar was used. When required, ampicillin was added to the medium to a final concentration of 100 μg/ml.

Purification of cytochrome c_3

S. frigidimarina NCIMB400 was grown at 23 °C without aeration in Luria–Bertani medium supplemented with 5 g/l NaCl and

20 mM fumarate. Cells were harvested at 4 °C; all subsequent purification steps were also performed at this temperature. Harvested cells (100 g wet weight) were resuspended in 100 mM sodium phosphate buffer, pH 7.0, and lysed by sonication. The lysate was centrifuged at 39000 g for 1 h and the supernatant was loaded on a DE-52 anion-exchange column (5 cm × 10 cm) that had been equilibrated with 100 mM sodium phosphate, pH 7.0. Cytochrome c_3 formed a tight band at the top of the column and was eluted with 100 mM phosphate buffer containing 200–250 mM NaCl. Protein fractions were pooled and concentrated before loading on a Sephadex G-50 column (2 cm × 150 cm) in 20 mM sodium phosphate buffer, pH 7.0. Further purification was achieved with a hydroxyapatite column (2.5 cm × 10 cm) with a gradient of 20–100 mM phosphate. The ratio of the Soret band at 407 nm over protein absorbance at 279 nm was used to monitor purity. Typical values of 12:1 were found. N-terminal sequencing was performed as described previously [17].

EPR spectroscopy

All measurements were made at 10 K with a Bruker ER 200D spectrometer with an Oxford Instruments cryostat and helium transfer system. Samples contained 0.2 mM protein in 100 mM sodium phosphate buffer, pH 7.0.

Redox potentials

Haem midpoint potentials were determined by protein-film voltammetry as described by Turner et al. [9]. Cytochrome c_3 was coated on a pyrolytic graphite-edge electrode, with polymyxin B as a co-adsorbate. Electrochemical studies were performed at 25 °C with a mixed buffer system of Taps, Hepes, Mes and Pipes, each at 50 mM, with 0.1 M NaCl as the supporting electrolyte. The buffers were titrated to the required pH with NaOH or HCl at 25 °C.

DNA techniques

Recombinant DNA techniques, Southern hybridization and genomic DNA library construction were performed as described by Sambrook et al. [18]. Dideoxy sequencing reactions [19] were performed with the Sequenase version 2.0 kit (USB). PCR was performed with *Tbr* thermostable polymerase (NBL Gene Sciences). Reactions typically consisted of 100 ng of *Shewanella* genomic DNA, 200 μM dNTPs and 1.5 mM $MgCl_2$ with the supplied reaction buffer; 20 ng of each oligonucleotide primer was present in each 50 μl reaction. Typically, two rounds of PCR were performed, with a 5 μl aliquot of the first-round reaction used as template in the second round. Cycles used were: 95 °C for 2 min; then 35 cycles of 95 °C for 20 s, 45 °C for 20 s and 72 °C for 10 s; and a final incubation at 72 °C for 5 min. To radiolabel DNA fragments for Southern hybridization experiments, [α-^{32}P]dCTP was included in the PCR reaction in place of unlabelled dCTP, and plasmid DNA containing cloned cytochrome c_3 PCR product was used as a template. DNA sequences were determined by the dideoxy chain termination method.

Degenerate primers

Primer sequences were designed on the basis of the N-terminal sequence of purified cytochrome c_3. The upstream primer (C31, 5′-CACGAATTCGARTTYCAYGTNGARATG-3′) contained an *Eco*RI recognition site and the codons for the sequence EFHVEM (single-letter amino acid codes). The downstream primer (C32, 5′-GCGAAGCTTYTTNARNGGYTCNCC-3′)

contained a *Hin*dIII recognition site and a sequence complementary to the codons for the amino acid sequence GEPSK.

Gene knock-out

The *cctA* gene encoding cytochrome c_3 was subcloned into pK18 within a 1.7 kb fragment and disrupted by inserting the spectinomycin/streptomycin resistance cassette, isolated as a *Hin*dIII fragment from pHRP310 [20] between the *Bsa*I and *Bst*XI sites, which flank the coding sequence, by blunt-end cloning. The disrupted *cctA* sequence was transferred from the resulting plasmid, pEG801, as a 3.4 kb *Sac*I–*Pst*I fragment to the suicide vector pJQ200KS [21] to give the recombinant plasmid pEG720. This was then transferred to *S. frigidimarina* NCIMB400 by conjugation as described by Gordon et al. [7] and disruptants were selected on the basis of their resistance to streptomycin and sucrose.

Growth in liquid culture used Luria broth supplemented with 15 mM formate and 50 mM Fe(III) citrate. Bottles were filled with this medium, leaving a minimal volume of gas, before being sealed and incubated at 23 °C. Cells were prepared for ferrozine assays by growth in the same medium but lacking Fe(III). Approximately 0.5 g wet weight of cells were washed in 50 mM Hepes, pH 7.0, and resuspended in the same buffer. The rate of Fe(III) reduction was measured in 2 ml Suba-sealed cuvettes containing 350 μM ferrozine and 500 μM formate in 50 mM Hepes, pH 7.0, and with 30–100 μl aliquots of cell suspensions. After 5 min of preincubation, reactions were initiated by the addition of Fe(III) citrate to 100 μM. The appearance of the Fe(II) ferrozine complex was monitored by measurements in triplicate of A_{562}.

RESULTS

Protein purification and characterization

When separating proteins from the periplasm of *S. frigidimarina* we observed an intense red band that bound very tightly to anion-exchange resins. This protein was purified from whole cell extracts by chromatography on anion-exchange and hydroxyapatite columns with a typical yield of 25 mg from 100 g cell wet weight. The isolated protein ran as a single band on SDS/PAGE and retained the haem group, despite the presence of SDS, indicating that the protein was a *c*-type cytochrome. The apparent molecular mass on SDS/PAGE was 15 kDa, but electrospray MS
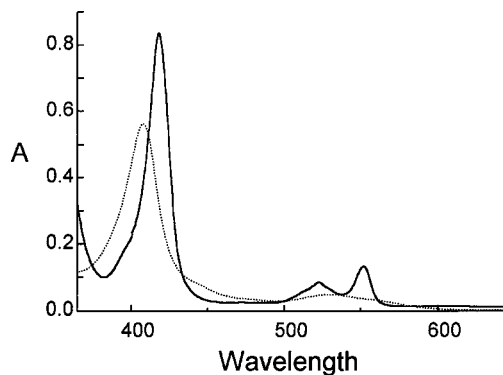

Figure 1   Absorbance spectra of *S. frigidimarina* cytochrome c_3

The protein (20 μg/ml in 10 mM Tris/HCl, pH 8.4) was reduced by the addition of dithionite (solid line) or oxidized by the addition of ferricyanide (dashed line); the absorbance was scanned at room temperature with a Shimadzu 1601 spectrophotometer.
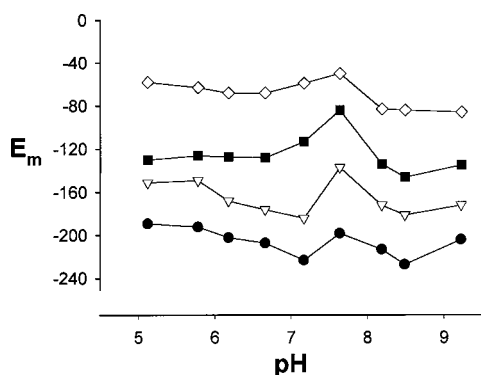
Figure 2   Midpoint potential values of cytochrome c_3 from *S. frigidimarina* NCIMB400 at 25 °C

Potentials were resolved for each of the four haems in experiments performed at several pH values. The error in each of the values given is approx. ± 15 mV.
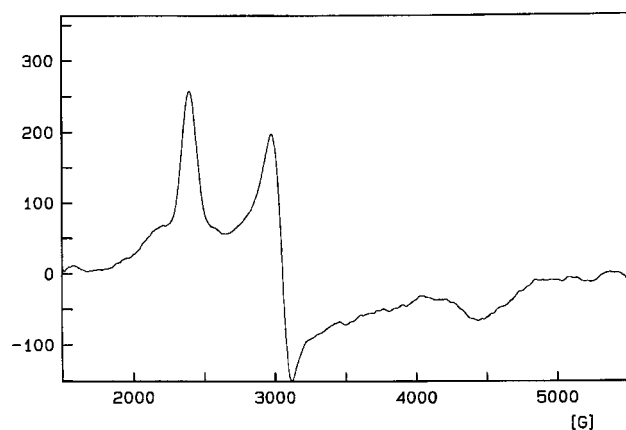

Figure 3   X-band EPR spectrum of oxidized *S. frigidimarina* cytochrome c_3, showing a single set of g values comprising $g_z = 2.83$, $g_y = 2.22$ and $g_x = 1.53$

These values are similar to those from bis-histidine-ligated haem groups in other cytochromes c_3.

indicated a molecular mass of 11780 Da, in close agreement with the value expected from the sequence as described below. We presume that the protein migrates anomalously in polyacrylamide gels because the conformation is constrained by the haem groups.

The purified protein exhibited typical absorbance spectra in the reduced and oxidized forms (Figure 1). Redox potentiometry of cytochrome c_3 by conventional methods showed that the haem groups titrated at low potential but individual haem potentials could not easily be resolved. Therefore purified cytochrome c_3 was subjected to protein-film voltammetry, permitting the resolution of each of the four haem midpoint potentials (Figure 2). The potentials were low, titrating in the range from -250 to 0 mV, with no significant dependence on pH. This method had also been used successfully with flavocytochrome c_3 from the same organism and the four haem groups of this protein titrated at similar potentials to those of cytochrome c_3 (-238, -196, -146 and -102 mV at pH 7.0) [9].

The EPR spectrum of oxidized cytochrome c_3 (Figure 3) indicates a single set of g values with $g_z = 2.83$, $g_y = 2.22$ and g_x

$= 1.53$. This behaviour is typical of bis-histidine-ligated haem groups.

The N-terminal amino acid sequence of purified cytochrome c_3 was determined for 26 cycles as ADETLAEFHVEMGG-EN-HADGEPSK. Cycles 15 and 18 were apparently blank, i.e. no amino acid residue was detected. This would be expected if the residues at these positions were cysteines involved in the covalent attachment of one of the haem groups.

Isolation of the gene encoding cytochrome c_3

The N-terminal amino acid sequence of the purified cytochrome c_3 was used to design degenerate oligonucleotide primers for PCR amplification of the coding sequence from *S. frigidimarina* genomic DNA. Restriction enzyme cleavage sites were included in each primer to facilitate the cloning of the resulting products. Two rounds of PCR were used to amplify the fragment of DNA encoding the N-terminus of the protein. A single product of the expected size (72 bp) was observed in the reactions. This band was excised from a 2% (w/v) agarose gel and purified with a Qiaex gel extraction kit. After digestion with *Eco*RI and *Hin*dIII, the DNA was repurified, again with Qiaex. The resulting DNA was then ligated into the vector pTZ19r [22], which had been cut with the same enzymes. Putative recombinant colonies [white on plates containing 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') and isopropyl β-D-thiogalactoside ('IPTG')] were isolated and the plasmid inserts were sequenced. Plasmid DNA from one of the positive clones was then used as a template to synthesize an [α-^{32}P]dCTP-labelled probe, which was then used to probe a Southern blot of *S. frigidimarina* DNA digested with various restriction enzymes. A positive hybridization signal was seen for 4 kb *Nsi*I-digested fragments. Bands of this size range were purified from a similar gel and cloned into *Pst*I-cut pTZ18r to form a library. From 800 screened colonies, two positive clones were isolated. The DNA sequence of the insert in one of the positive recombinant plasmids, pEG700, was then determined (Figure 4).

Sequence analysis

The coding sequence of the *cctA* gene is 272 bp, including a putative signal sequence of 25 residues. Three consecutive ATG codons were found preceding the sequence encoding the N-terminus of purified cytochrome c_3. However, the predicted sequence from any of these as the initiation codon did not fit the pattern for N-terminal secretory signal sequences that are characteristic of periplasmic proteins. It seems most likely that translation was initiated at a GTG codon (Figure 4), predicting a typical signal sequence of 25 residues with a basic N-terminus and a long hydrophobic segment [23]. A putative ribosome-binding site (in capitals in Figure 4) was located just 9 bp away from this GTG codon, consistent with this being the site of translation initiation.

Examination of the DNA sequence immediately downstream of the translational stop codon revealed a region with similarity to bacterial rho-independent transcriptional terminators, implying that the *cctA* gene was not co-transcribed with any other coding sequence. Indeed, Northern blot hybridization analysis of total RNA extracted from *S. frigidimarina* cells suggested that the *cctA* mRNA was in the range of 400–600 bp in length (results not shown).

Sequencing downstream of the *cctA* gene revealed a coding sequence in the same orientation as *cctA* that encoded a putative protein product with extensive similarity to nitrate and formate reductases, both of which contain a molybdenum cofactor. This reading frame was particularly closely related to the assimilatory

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  1 tattgtcagttaatttgagattgtttataaatctttgataaacggtttataaacaaagtt

 61 gttgaataacccttaagcaaagtgtcgtgttgcagtgtgatgatccgcgtcagataaaag

                                        M  S  N  K  L  L  S  A  L
121 aatgcggacacacactaaattGGAGGaatgaatagtgagcaataaaactactaagtgcatt

     F  A  A  G  F  A  V  M  M  M  S  S  A  S  F  A  A  D  E  T
181 gtttgcggctggtttcgcggtaatgatgatgtcttctgcatcatttgctgctgatgagac

     L  A  E  F  H  V  E  M  G  G  C  E  N  C  H  A  D  G  E  P
241 cctcgcagagtttcacgttgaaatgggtggctgtgaaaactgtcacgctgatggtgaacc

     S  K  D  G  A  Y  E  F  E  Q  C  Q  S  C  H  G  S  L  A  E
301 atcaaaagatggcgcttatgaatttgaacaatgtcaaagttgtcatggttcactagctga

     M  D  D  N  H  K  P  H  D  G  L  L  M  C  A  D  C  H  A  P
361 aatggatgataaccataagccacatgatgggttacttatgtgtgctgattgtcatgcgcc

     H  E  A  K  V  G  E  K  P  T  C  D  T  C  H  D  D  G  R  T
421 acatgaagcaaaagtaggcgaaaagccaacatgtgatacatgccacgatgatggccgtac

     A  K  *
481 tgcaaaataagttatcttagatagcttgaaaataccgacataatgtcggtattttgttt

541 ttattcctcaagagtatacatctcacttttatttttatacctctttataggtatttaaag
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Figure 4   DNA sequence of the _cctA_ gene from _S. putrefaciens_

The inferred protein sequence is shown above the DNA sequence. Underlined amino acids indicate those determined by N-terminal sequencing of purified cytochrome c_3. A putative ribosome-binding site is shown in capitals; DNA sequences similar to a rho-independent terminator are overlined.

nitrate reductases (41 % identity with a cyanobacterial sequence) (Figure 5A), which are cytoplasmic enzymes involved in the utilization of nitrate as a nitrogen source. The nitrate reductase initiation codon was 285 base pairs downstream from the cytochrome c_3 gene termination codon. Upstream of _cctA_ was a further reading frame in the same orientation as the _cctA_ gene. This showed extensive similarity to 3-hydroxyisobutyrate de-hydrogenases (31 % identical with the _E. coli_ sequence) (Figure 5B) which, in bacteria, are cytoplasmic, NAD$^+$-dependent enzymes required for valine catabolism. There was a gap of 395 bp between this reading frame and the cytochrome c_3 coding sequence. These sequences are not shown here but are included in the EMBL database entry. The observation that the cyto-chrome c_3 gene was flanked by coding sequences clearly unrelated to anaerobic respiration indicates that _cctA_ is likely to be transcribed as a monocistronic RNA.

Predicted sequence of cytochrome c_3

The mature cytochrome c_3 consists of only 86 amino acid residues with a predicted molecular mass of 9316 Da excluding the haem groups. The attachment of four haems would result in a molecular mass of 11780 Da; this was the value obtained by MS of the purified protein. Cytochrome c_3 is an extremely acidic protein with a net charge of -14 (10 Asp, 10 Glu, 5 Lys and 1 Arg). In the fully oxidized state the holoprotein has a net charge of -10. The eight Cys and eight His residues are presumably all involved in haem attachment and ligation.

The cytochrome c_3 amino acid sequence was compared with sequences in the available databases. The only clear similarity was seen to other proteins from _Shewanella_ or closely related organisms: the cytochrome domains of the two _S. frigidimarina_ flavocytochromes c_3 [4,24], the related flavocytochrome c_3 from _S. oneidensis_ MR-1 [25] and cytochromes c_3 from _S. oneidensis_

Figure 5   Alignments of the polypeptide sequences predicted by the reading frames flanking the cctA sequence with close relatives

(**A**) The reading frame downstream of _cctA_ (bottom row of each pair) aligned with the sequence of nitrate reductase from _Synechococcus_ sp. (top row of each pair; Swissprot entry NARB-SYNP7 [31]). (**B**) The reading frame upstream of _cctA_ (bottom row) aligned with the sequence of 3-hydroxyisobutyrate dehydrogenase from _Escherichia coli_ (top row of each pair; Swissprot entry YHAE-ECOLI [32]). The alignments were produced with ClustalW.

MR-1 [26] and the organism H1R [27]. These sequences are aligned in Figure 6 and show a considerable degree of similarity. The relationship with cytochromes c_3 from _Desulfovibrio_ species is much more distant and no significant similarity is observed outside the haem attachment sites (CXXCH) conserved in all c-type cytochromes.

Gene disruption

No clues to the function of cytochrome c_3 were obtained from its location in the genome (it is not encoded within an operon); we therefore constructed a null mutant to examine the phenotypic consequences of the lack of this protein. This was achieved by replacing the cytochrome c_3 coding sequence of _S. frigidimarina_ NCIMB400 with a streptomycin/spectinomycin resistance cassette by homologous recombination. The presence of the expected gene disruption was verified by Southern blot hybrid-ization; the ability of the resultant strain, AH301, to utilize a range of electron acceptors for anaerobic respiration was de-termined by following growth on plates and in liquid culture. The ability of AH301 to utilize nitrate, nitrite, trimethylamine _N_-oxide, DMSO, fumarate, tetrathionite and sulphite were indistinguishable from that of the parent strain, indicating that

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Shf    -ADETLAEFHVEMGGCENCHADG---EPSKDGAYEFEQCQSCHGSLAEMDD--N------  48
H1R    --ADVLADMHAEMSGCETCHADG---APSEDGAHEAAACADCHGGLADMEA--P------  47
Fcc    --ADNLAEFHVQNQECDSCHTPD-GELSNDSLTYENTQCVSCHGTLAEVAETTKHEHYNA  57
Ifc    -----MGSFHADMGSCQSCHAKP-IKVT-DSETHENAQCKSCHGEYAELAN--DKLQFDP  51
Mrf    AAPEVLADFHGEMGGCDSCHVSDKGGVTNDNLTHENGNCVSCHGDLKELAAAAPKDKVSP  60
MR1    -ADQKLSDFHAESGGCESCH---                                       19


Shf    HKPHDG-LLMCADCHAPHEAKVGEKPTCDTCHDDGRTAK-------  86
H1R    HPAHDG-MLECTDCHMMHEDEVGSRPACDACHDDGRTA--------  84
Fcc    HASHFPGEVACTSCHSAHEKSM---VYCDSCHSFDFN-MPYAKK---  97
Ifc    HNSHLG-DINCTSCHKGHEEPK---FYCNECHSFDIKPMPFSDAKKK  94
Mrf    HKSHLIGEIACTSCHKGHEKSV---AYCDACHSFGFD-MPFGGK--- 100
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Figure 6   **Alignment of amino acid sequences of cytochrome c_3 from *S. frigidimarina* (Shf) with the corresponding protein from the phototroph H1R (H1R), partial sequences of flavocytochrome c_3 and its iron-induced isoenzyme from *S. frigidimarina* (Fcc and Ifc respectively) and the flavocytochrome c_3 from *S. oneidensis* MR-1 (Mrf) corresponding to the tetrahaem cytochrome c domain and with the N-terminal sequence of cytochrome c_3 from *S. oneidensis* MR-1 (MR1)**

The haem attachment sites (CXXCH) and the histidine ligands to the haem irons are shown in bold.
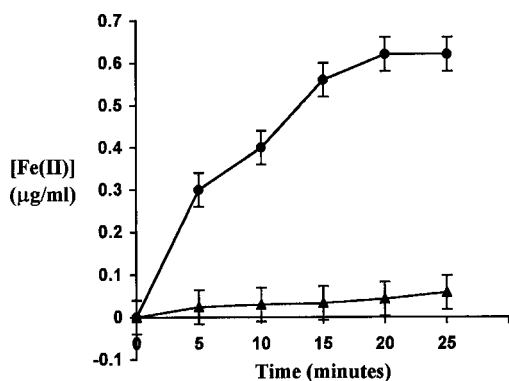

Figure 7   **Reduction of Fe(III) by *S. frigidimarina* NCIMB400 and AH301(Δc_3)**

Cells grown anaerobically were washed in 50 mM Hepes, pH 7.0, and resuspended in the same buffer. Equivalent aliquots from NCIMB400 (●) and AH301 (▲) were mixed anaerobically with 50 mM Hepes containing 500 μM formate and 100 μM Fe(III) citrate. The increase in A_{562} was followed and used to calculate the Fe(II) concentration. Measurements were performed in triplicate.

cytochrome c_3 is not required for electron transfer to these oxidants. In contrast, growth with Fe(III) citrate was clearly impaired. After an anaerobic incubation for 2 weeks at 23 °C in medium containing 15 mM formate and 50 mM iron (III) citrate, the parent strain, NCIMB400, grew well and completely reduced the iron to Fe(II), whereas the mutant AH301 substantially failed to dissimilate the iron, as judged by the colour of the medium. Growth of the mutant was greatly impaired, with the maximal attenuance being less than 35 % of that achieved by the wild-type parent strain. This level of growth might not have required Fe(III) reduction because a small amount of yeast extract was added; growth was extremely slow if this was omitted. To measure Fe(III) reduction more directly, we grew cultures anaerobically in the absence of Fe(III); the ability of whole cells to reduce Fe(III) was then determined spectro-photometrically with a ferrozine assay to detect Fe(II) production (Figure 7). Ferrozine forms a complex with Fe(II), with a strong absorbance maximum at 562 nm. These assays clearly showed that deletion of the cytochrome c_3 gene severely impairs the

ability of *S. frigidimarina* to reduce Fe(III), although a low level of Fe(II) production remained in AH301.

DISCUSSION

During anaerobic growth, *S. frigidimarina* synthesizes several cytochromes that are absent when O_2 is available. One of these is a small acidic cytochrome that we have shown to contain four haem groups on the basis of absorption coefficients and the presence of four typical c-type haem attachment sites (CXXCH) in the predicted amino acid sequence. This protein shares several properties, including midpoint reduction potentials and bis-histidine ligation, with the cytochromes c_3 from sulphate reducers. We have therefore classified the *S. frigidimarina* protein also as a cytochrome c_3.

The sequence of the *cctA* gene encoding cytochrome c_3 failed to provide further clues to the function of this protein. Many respiratory proteins in bacteria are encoded in operons and are co-expressed with functionally related proteins. The cytochrome c_3 from *S. frigidimarina* NCIMB400 is produced from a small monocistronic RNA and the *cctA* gene is flanked by sequences encoding cytoplasmic enzymes with functions unrelated to anaerobic electron transfer.

The low reduction potential of this protein and its production only during anaerobiosis indicate that it is most probably involved in one or more pathways of anaerobic respiration. To address its possible function we constructed a cytochrome c_3 null mutant and showed that it has a greatly impaired ability to reduce Fe(III) to Fe(II), indicating that cytochrome c_3 is involved in the electron transfer to this respiratory oxidant. Other components of this pathway remain to be characterized, although an outer-membrane decahaem cytochrome c has been identified in the related freshwater *Shewanella*, *S. oneidensis* MR-1, as a component of an operon that is required for Fe(III) respiration [28], as has the inner-membrane tetrahaem cytochrome, CymA [29]. It is possible that cytochrome c_3 shuttles electrons across the periplasm between these two proteins.

A small cytochrome c_3 has also been isolated from *S. oneidensis* MR-1 (previously *S. putrefaciens* [30]). The spectroscopic and redox properties of this protein are similar to those of the cytochrome c_3 from *S. frigidimarina* NCIMB400 but the N-terminal sequences [26] show considerable divergence (Figure 6). These sequence differences could reflect different functions for these two proteins or might simply be indicative of a rather distant relationship between the two *Shewanella* strains, perhaps reflected in their different habitats. Furthermore, the genetic context of the cytochrome c_3-coding sequence in the two *Shewanella* species is quite different. In *S. frigidimarina* it is flanked by genes apparently encoding 3-hydroxyisobutyrate dehydrogenase and an assimilatory nitrate reductase, whereas in MR-1 the flanking genes encode homologues of HtpX heat-shock protease and a hydrogenase cytochrome b subunit. The MR-1 DNA sequence is available at www.tigr.org and this organism seems to encode only one genuine homologue of the cytochrome c_3 from *S. frigidimarina*.

The close relationship between cytochrome c_3 and the cyto-chrome domain of flavocytochrome c_3 from the same organism, *S. frigidimarina* NCIMB400, indicates a relatively recent dupli-cation of this sequence. No organisms other than *Shewanella* spp. have been shown to contain a flavocytochrome c_3 type of fumarate reductase and it is probable that this protein arose by the fusion of an *FrdA* sequence (encoding the flavoprotein subunit of a typical membrane-bound bacterial fumarate re-ductase) with a cytochrome c_3-coding sequence. The physical and spectroscopic properties of cytochrome c_3 and the cyto-

chrome domain of flavocytochrome c_3 are very similar but their functions are quite different. We have shown previously by gene disruption that flavocytochrome c_3 is clearly required for fumarate reduction but not for other electron transfer pathways [7]. The small cytochrome c_3 is, in contrast, involved in electron transfer to Fe(III).

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