Physiological function and regulation of flavocytochrome c_3 , the soluble fumarate reductase from *Shewanella putrefaciens* NCIMB 400

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***Shewanella putrefaciens* produces a soluble flavocytochrome c under anaerobic growth conditions. This protein shares sequence similarity with the catalytic subunits of membrane-bound fumarate reductases from *Escherichia coli* and other bacteria and the purified protein has fumarate reductase activity. It is shown here that this enzyme, flavocytochrome c_3 , is essential for fumarate respiration *in vivo* since disruption of the chromosomal *fccA* gene, which encodes flavocytochrome c_3 , leads to a specific loss of the ability to grow with fumarate as terminal electron acceptor. Growth with nitrate, trimethylamine N -oxide (TMAO) and other acceptors was unaffected. The *fccA* gene is transcribed as a 2 kb monocistronic mRNA. An adjacent reading frame that bears limited sequence similarity to one of the membrane anchor subunits of *E. coli* fumarate reductase is not co-transcribed with *fccA*. Expression of the *fccA* gene is regulated by anaerobiosis and by the availability of alternative electron acceptors, particularly nitrate and TMAO. DNA sequences have been identified that are required for this regulation.**

Keywords: *Shewanella*, fumarate reductase, flavocytochrome, electron transfer, anaerobic respiration

INTRODUCTION

The Gram-negative facultative anaerobe *Shewanella putrefaciens* exhibits an enormous diversity of metabolism that is particularly apparent during anaerobic respiration. In the absence of oxygen, *S. putrefaciens* can couple growth to the reduction of at least 10 different terminal electron acceptors, including nitrate, nitrite, fumarate, thiosulphate, elemental sulphur (S^0), trimethylamine N -oxide (TMAO) and, more unusually, the particulate metal oxides Mn(IV) and Fe(III) (Myers & Nealson, 1988; Moser & Nealson, 1996).

Anaerobic growth of *S. putrefaciens* with fumarate as the terminal electron acceptor results in the synthesis of several novel cytochromes (Morris *et al.*, 1990), the most abundant of which is a soluble, periplasmic, tetrahaem

flavocytochrome c (Fcc₃; 63·8 kDa) that has been shown biochemically to be a unidirectional fumarate reductase (Pealing *et al.*, 1992). The gene encoding Fcc₃ has been cloned and sequenced (Pealing *et al.*, 1992). From the inferred amino acid sequence, Fcc₃ can be seen to consist of two domains: a small N-terminal cytochrome domain, and a larger C-terminal flavoprotein domain containing non-covalently bound FAD.

Other bacterial fumarate reductases are membrane-bound multisubunit enzymes consisting of two membrane extrinsic subunits (a flavoprotein and an iron-sulphur polypeptide) anchored to the inner face of the cytoplasmic membrane by either one or two membrane anchors, depending on the source organism (Ackrell *et al.*, 1992). Sequence analysis clearly shows that the flavin domain of Fcc₃ is related to the flavoprotein subunits of the membrane-bound fumarate reductases and succinate dehydrogenases (Pealing *et al.*, 1992). The N-terminal cytochrome domain of Fcc₃ contains four covalently bound haem groups and shows only limited similarity to cytochrome c_3 from sulphate reducers. We have recently

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Abbreviation: TMAO, trimethylamine N -oxide.

purified a low-potential cytochrome c_3 from *S. putrefaciens* and the gene encoding this protein has now been cloned and sequenced (E. Gordon, A. Pike, H. Fischer, S. Chapman & G. Reid, unpublished). In light of the similarity between this and other cytochromes c_3 to the haem domain of Fcc₃, we propose that the *Shewanella* fumarate reductase should be referred to as flavocytochrome c_3 (Fcc₃) to avoid possible confusion with other flavocytochromes c , such as the sulphide dehydrogenase from *Chromatium vinosum* (Dolata *et al.*, 1993) and *p*-cresol methylhydroxylase (Kim *et al.*, 1994), which are quite different from Fcc₃ in both structure and function.

Although Fcc₃ catalyses fumarate reduction *in vitro*, its physiological role has not previously been determined. Indeed, *S. putrefaciens* MR-1 has been reported to contain two fumarate reductases on the basis of both cell fractionation studies (Myers & Myers, 1992) and ESR spectroscopy with whole cells (Tsapin *et al.*, 1995). The latter study identified resonances that were attributed to iron-sulphur centres from a typical membrane-bound fumarate reductase whereas cell fractionation allowed separation of two soluble fumarate reductase activities, one cytosolic and one periplasmic. The latter of these is presumably flavocytochrome c_3 .

Fcc₃ is anaerobically induced (Pealing *et al.*, 1992) but the mechanism for this regulation is unknown. *S. putrefaciens* contains a homologue of the *Escherichia coli* FNR protein (Saffarini & Nealson, 1993). This protein, EtrA, is thought to be responsible for global regulation in response to anaerobiosis. Strains of *S. putrefaciens* that are deficient in EtrA are unable to respire on nitrite, TMAO, DMSO, thiosulphate and fumarate (Saffarini & Nealson, 1993), indicating that some essential component of these pathways is regulated by EtrA. In *E. coli*, the *frd* operon encoding fumarate reductase is positively regulated by FNR. Given that known members of the FNR family of proteins have well-conserved target sites (the predicted DNA-binding domains of EtrA and FNR are identical, and overall the proteins exhibit 73.6% sequence identity) and, most importantly, that EtrA functionally complements an *E. coli* *fnr* null mutant (Saffarini & Nealson, 1993), it is likely that the EtrA target site will be very similar, if not identical, to the *E. coli* FNR DNA target sequence. However, only rather weak matches to the consensus FNR-binding site (Spiro, 1994) are apparent in the region upstream from the Fcc₃ coding sequence. We have therefore attempted to identify regions responsible for anaerobic regulation by measuring expression of *lacZ* under control of modified forms of the *fccA* promoter.

To investigate the role of Fcc₃ in anaerobic respiration, we have constructed a null mutant of *fccA* and examined the phenotypic consequences of a lack of Fcc₃. We have also investigated the regulation of the *fccA* gene and examined promoter sequence requirements for the regulation of *fccA*.

METHODS

Media, strains and plasmids. *S. putrefaciens* and *E. coli* strains are listed in Table 1. *S. putrefaciens* strains were routinely grown at 23 °C and *E. coli* at 37 °C. Luria broth or agar was used for growth of all strains. Minimal medium for growth of *S. putrefaciens* strains was as described by Myers & Nealson (1988) except for the addition of 0.2 M NaCl (Lee *et al.*, 1977) and 20 mM succinate. Antibiotics were used at the following concentrations: kanamycin, 50 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; streptomycin, 25 µg ml⁻¹ (pHRP-based constructs) or 100 µg ml⁻¹ (pMMB-based constructs); gentamicin, 10 µg ml⁻¹; rifampicin, 10 µg ml⁻¹. IPTG was used at a concentration of 500 µg ml⁻¹. Plasmids used are listed in Table 2.

RNA manipulations. Total RNA was purified as described by Ausubel *et al.* (1993). Primer extensions were carried out as described by Sawers & Böck (1989) except that M-MLV reverse transcriptase (BRL) was used with the primer 364P (5'-GTGAAGTCGGATTGATT-3'), which is complementary to the DNA sequence 63–79 bp upstream from the translational start codon, and primer G4300 (5'-CGTCAGACATAACGATGT-3'), which is complementary to codons 7–12 of the Fcc₃ signal sequence (Pealing *et al.*, 1992). S1 nuclease analysis was carried out using published protocols (Ausubel *et al.*, 1993). The 564 bp *Bss*HII–*Nsi*I fragment encompassing the putative termination site was end-labelled using Klenow fragment and [α -³²P]dCTP at the *Bss*HII restriction site, resulting in the labelling of the complementary strand. Northern blotting and hybridization were carried out as described by Ausubel *et al.* (1993). Hybond-N (Amersham) membranes were used throughout for blotting.

DNA manipulations and Southern blotting. DNA manipulations were carried out as described in Sambrook *et al.* (1989). Restriction and DNA-modifying enzymes (Promega, NBL, NEB) were used according to the manufacturers' protocols. Site-directed mutagenesis was carried out as described by Kunkel & Roberts (1987). *S. putrefaciens* genomic DNA was prepared as per Méjean *et al.* (1994). Southern blot hybridization was carried out in 6 × SSC, 1 × Denhardt's solution at 65 °C. DNA sequencing was carried out using the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 kit (United States Biochemical).

Deletion of *fccA* and *orf2*. The 3.3 kb *Sac*I–*Pst*I fragment containing *fccA* and part of the *orf2* sequence was ligated into pEG300 (this plasmid is identical to pTZ19r except that the *Eco*RI site has been removed) that had been cut with *Sac*I and *Pst*I to form pEG301. pEG301 was then digested and re-ligated to remove the 1705 bp sequence between the two *Eco*RI sites to form pEG302. The *ahp* gene from pRS551 was excised on a 1340 bp *Stu*I fragment and ligated into pEG302 that had been cut with *Eco*RI and end-filled with T4 DNA polymerase to give pEG303. The *fccA::ahp* construct was excised on a 2.7 kb *Sac*I–*Pst*I fragment and ligated into pJQ200KS (Quandt & Hynes, 1993) cut with the same enzymes. This final plasmid, pEG304, was used for all subsequent mating experiments. Gene replacement was carried out exactly as by Quandt & Hynes (1993).

β -Galactosidase measurement. Cultures were analysed in mid-exponential phase as described by Miller (1972). Each data point represents at least three independent experiments. Individual values rarely varied by more than 10%.

Construction of expression cassettes. Different forms of Fcc₃ were expressed by introducing *Eco*RI and *Hind*III cleavage sites at appropriate places in the coding sequence. The following oligonucleotides were used for site-directed muta-

Table 1. Bacterial strains used

Strain	Characteristics	Source/reference
<i>Shewanella putrefaciens</i>		
NCIMB 400	Wild-type <i>S. putrefaciens</i>	NCIMB
NCIMB 400R	Spontaneous Rif ^r strain	Laboratory stock
EG301	NCIMB 400 Rif ^r $\Delta fcc::ahp$ Km ^r	This study
EG402	NCIMB 400 Rif ^r $\Delta orf2::ahp$ Km ^r	This study
EG281	NCIMB 400 Rif ^r $orf2::(ahp T1_4 fcc' lacZ)$ transcriptional	This study
EG282	NCIMB 400 Rif ^r $orf2::(ahp T1_4 fcc' 'lacZ)$ translational	This study
EG322	NCIMB 400 Rif ^r $orf2::(ahp T1_4 lacZ)$	This study
EG323	NCIMB 400 Rif ^r $orf2::(ahp T1_4 'lacZ)$	This study
<i>Escherichia coli</i>		
MC4100	<i>araD139</i> $\Delta(argF-lac)U169$ <i>flbB53 ptsF25 relA1 rpsL150 deoC1</i> l ⁻ F ⁻	Casadaban & Cohen (1979)
DH5 α	<i>supE44</i> $\Delta lacU169$ ($\phi 80 lacZ \Delta M15$) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco-BRL
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> $\Delta(lac-proAB)$ F'[<i>traD36 proAB⁺ lacI^q lacZ</i> $\Delta M15$]	Sambrook <i>et al.</i> (1989)
JM110	<i>dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx</i> $\Delta(lac-proAB)$ F'[<i>tra D36 proAB⁺ lacI^q lacZ</i> $\Delta M15$]	NEB
S17-1	<i>thi pro hsdR hdsM⁺ recA</i> , chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7)	Simon <i>et al.</i> (1983)
SM10	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^R</i>	Simon <i>et al.</i> (1983)
BW313	<i>dut ung thi-1 relA spoT1/F' lysA</i>	Kunkel & Roberts (1987)

Table 2. Plasmids used in this study

Plasmid	Description	Source/reference
pHRP309	Broad-host-range transcriptional fusion vector Gm ^r , <i>lacZ</i>	C. Harwood, University of Iowa
pJQ200KS	Mobilizable direct selection Gm ^r	Quandt & Hynes (1993)
pEG200	pHRP309 Ω Sm 773 bp upstream of <i>fccA</i> translational start codon	This work
pEG210	pHRP309 Ω Sm 564 bp upstream of <i>fccA</i> translational start codon	This work
pEG644	pHRP309 Ω Sm 319 bp upstream of <i>fccA</i> translational start codon	This work
pEG648	pHRP309 Ω Sm 254 bp upstream of <i>fccA</i> translational start codon	This work
pEG216	pHRP309 Ω Sm 101 bp upstream of <i>fccA</i> translational start codon	This work
pEG675	pHRP309 Ω Sm 308 bp upstream of <i>fccA</i> translational start codon	This work
pEG676	pHRP309 Ω Sm 304 bp upstream of <i>fccA</i> translational start codon	This work
pEG677	pHRP309 Ω Sm 285 bp upstream of <i>fccA</i> translational start codon	This work
pEG678	pHRP309 Ω Sm 274 bp upstream of <i>fccA</i> translational start codon	This work
pEG622	pHRP309 Ω Sm 753 bp <i>fccA KpnI-SnaBI</i>	This work
pEG637	pHRP309 Ω Sm 672 bp <i>fccA KpnI-DraI</i>	This work
pEG638	pHRP309 Ω Sm 454 bp <i>fccA KpnI-HpaI</i>	This work
pEG304	pJQ200KS <i>fccA::ahp</i>	This work
pEG403	pJQ200KS <i>orf2::ahp</i>	This work
pMMB503EH	Expression vector, <i>tac</i> promoter Sm ^r	Michel <i>et al.</i> (1995)
pEGX1	pMMB503EH <i>fccA</i> with signal sequence	This work
pEGX8	pMMB503EH <i>fccA</i> without signal sequence	This work
pTZ19r	Cloning vector	Mead <i>et al.</i> (1986)
pEG281	pJQ200KS $orf2::(ahp T1_4 fccA' lacZ)$	This work
pEG282	pJQ200KS $orf2::(ahp T1_4 fccA' 'lacZ)$	This work
pEG681	pJQ200KS $orf2::(ahp T1_4 lacZ)$	This work
pEG682	pJQ200KS $orf2::(ahp T1_4 'lacZ)$	This work
pRS551	Ap ^r Km ^r <i>lacZ lacY⁺ lacA⁺</i>	Simons <i>et al.</i> (1987)
pRS552	Ap ^r Km ^r ' <i>lacZ, lacY⁺ lacA⁺</i>	Simons <i>et al.</i> (1987)

genesis by the Kunkel method (Kunkel & Roberts, 1987): 917R (GGGAGCAAAAGAATTCATGAAAAAGATG), which inserts an *EcoRI* site at the start of the signal sequence; 918R (ATGGGCACTGAATTCATGGCTGATAAC), which inserts an *EcoRI* site and an ATG codon at the start of the mature *Fcc₃* coding sequence; 920R (AAAAGAACTAAAA-GCTTTAGAATTC), which inserts a *HindIII* site at the end of the *Fcc₃* coding sequence; 921R (GCTGAATTCATGTACAA) and 922R (GTCGCAAACTTGATCC), which remove internal *EcoRI* and *HindIII* restriction sites, respectively, but do not alter the protein sequence. To construct a cassette for expression of the flavoprotein domain, primers 919R (GAATTGGCCGAATTCATGTCAGAACGTC), which inserts an *EcoRI* site and an ATG codon at the start of the flavin domain, and *Fcc100* (GCTGATAACTTAGCTA-GCGCACCTC), which deletes the cytochrome domain coding sequence from *fccA*, were used. This results in the attachment of the *Fcc₃* signal sequence to the flavin domain sequence.

Construction of single copy fusions. Essentially the single copy transcriptional and translational fusions consisted of the 6114 bp and 6014 bp *DraI* restriction fragment from pRS551 and pRS552, respectively (Simons *et al.*, 1987), with the 785 bp *EcoRI*–*HinFI* *fccA* restriction fragment (the putative *fccA* gene promoter) ligated in front of *lacZ*. In the case of the translational fusion construct, an in-frame fusion was generated and this was verified by direct sequencing of the resultant plasmid. These constructs consisted of the *ahp* gene, the ribosomal terminator sequences and the *lacZ* gene, inserted into the *orf2* sequence which had been cut with *PstI* and *Clal*. Finally the entire constructs were transferred to pJQ200KS and used to 'gene replace' *orf2*, which resulted in a single copy fusion assay system. Positive colonies were initially screened as blue on X-Gal, Km^r, sucrose-resistant, Ap^r, Gm^r. Putative fusion strains were checked for intact *fccA* sequence by Southern blot hybridization, and for the presence of *Fcc₃* by Western blotting.

RESULTS

Northern blot analysis of *fccA*

To initially characterize the pattern of expression of the *fccA* gene we examined the transcripts produced in *S. putrefaciens* NCIMB 400 that had been grown either aerobically or anaerobically with fumarate as terminal electron acceptor. A 1705 bp *EcoRI* fragment (Fig. 1) internal to the *fccA* gene was used as a probe in a Northern blot hybridization experiment (Fig. 2) to analyse the *fccA* specific transcripts produced under

Fig. 1. Physical map of the *fccA* gene and flanking DNA sequences. *fccA*, *orf1* and *orf2* denote the structural genes for *Fcc₃*, *Orf1* and *Orf2*, respectively. The arrows indicate the direction of transcription for each of these reading frames. The location on the 1.7 kb *EcoRI* fragment that was used to probe the Northern blot is indicated.

Fig. 2. Northern blot analysis of *fccA* transcription. Total cellular RNA (5 µg) prepared from exponentially growing cultures of *S. putrefaciens* NCIMB 400, grown aerobically (lane 1) or anaerobically (lane 2) in LB medium. The medium was supplemented with 20 mM lactate and 20 mM fumarate for anaerobic growth. The positions of size markers are indicated.

aerobic and anaerobic conditions. A positive hybridization signal was seen for RNA samples prepared from *S. putrefaciens* grown anaerobically with fumarate but no corresponding signal was seen for the RNA sample prepared from aerobically grown cells. Faint hybridization was observed at a lower apparent size in both RNA samples but no bands were seen that migrated more slowly on the gel. This positive signal corresponds to an *fccA* mRNA of ~2000 bp. In a previous paper, we pointed out the moderate similarity between the reading frame immediately downstream from, and in the same orientation as, the *fccA* coding sequence and *Frdd*, which is one of the membrane anchor subunits of *E. coli* fumarate reductase (Pealing *et al.*, 1992). This led us to consider the possibility that these two coding sequences might be co-transcribed. An RNA resulting from the co-transcription of *fccA* and *orf2* would be expected to be at least 2620 bp long; no transcripts of this size were detected.

Determination of the 5'- and 3'-ends of the *fccA* transcript

The location of the 5'-end of the *fccA* mRNA was determined using primer extension. These experiments showed that *fccA* transcription initiates 151 nucleotides upstream of the ATG initiation codon of *fccA* (Fig. 3a). Analysis of the DNA sequence upstream of the transcription start site revealed a possible -10 sequence, 5'-AAAGAT-3', which matches (underlined) three of six bases with the *E. coli* consensus sequence (5'-TATAAT-3') for σ^{70} -specific promoters. There is a possible -35 element of the promoter, 5'-TTGTTG-3', matching three of six bases of the *E. coli* consensus sequence (5'-TTGACA-3') and the -10 and -35 elements are 17 bases apart. No obvious matches to the consensus FNR target site (5'-TTGATN₄ATCAA-3') were found in the DNA sequences upstream of the coding region. The closest matches were found centred at -25.5 relative to the transcription start site (5'-TTGATtgcgCCCC-3'), which overlaps the putative -35 sequence, and another centred at -153.5 (5'-GTGATctatCTCCA-3'). Both of

Fig. 3. Determination of the 5'- and 3'-ends of the *fccA* mRNA transcript. Total RNA was prepared from anaerobically grown *S. putrefaciens* in LB medium supplemented with 20 mM lactate and fumarate, respectively. (a) Primer extension analysis of the 5'-end of the *fccA* mRNA. Dideoxy sequencing reactions generated from the same primer were used as size standards. The DNA sequence read from this autoradiograph is shown to the right, along with its complement which corresponds to the sequence of the coding strand (reading top to bottom). (b) S1 nuclease mapping of the 3'-end of the *fccA* mRNA transcript. Dideoxy sequencing reactions were used as size standards for the reaction.

these are outwith the usual locations of -40 to -50 relative to the transcription start site for promoters activated by FNR and, although these putative EtrA sites show a degree of sequence similarity to the consensus *E. coli* FNR target sequence, this is pronounced only in the first half-site.

The 3' end point of *fccA* mRNA

The location of the 3'-end of the *fccA* transcript was determined using high-resolution S1 nuclease mapping. A 565 bp *Bss*HII–*Nsi*I restriction fragment (Fig. 1) was used as a probe for S1 analysis. This fragment encompassed the last 85 codons of the *fccA* gene, and a further 310 bp beyond the translational stop codon to the *Nsi*I site preceding the *orf2* gene. Using this fragment labelled with [α - 32 P]dCTP at the *Bss*HII restriction site and total

RNA prepared from *S. putrefaciens* NCIMB 400 that had been grown anaerobically with fumarate, S1 nuclease analysis revealed a protected fragment of 314 bp (Fig. 3b). A protected fragment of this size would correspond to a transcript which terminated at a cytosine, 60 bp downstream from the *fccA* translational stop codon. Analysis of the putative mRNA sequence 3' to the *fccA* coding sequence for possible terminator sequences revealed an inverted repeat followed by a run of five uracil residues. These features are characteristic of rho-independent terminators (Richardson, 1993).

Regulation of *fccA* expression

To investigate the regulation of the *fccA* gene, single copy operon (EG281) and protein (EG282) fusions between the *fccA* promoter and *lacZ* were constructed. The effects of various terminal electron acceptors on the expression of *fccA*–*lacZ* transcriptional and translational fusions were then monitored by assaying the β -galactosidase levels in permeabilized cells of EG281 and EG282 grown with different respiratory oxidants (Table 3). Growth with nitrate resulted in a 35-fold decrease in activity and oxygen caused a 76-fold decrease in the β -galactosidase activity in the fusion strains. The expression of *fccA* also appeared to be regulated in response to TMAO, the presence of which caused an 18-fold decrease in β -galactosidase activity. The consistent difference (~ 2.5 -fold) between the operon and protein fusion activity levels was probably due to differing translational efficiencies between the operon (which uses an *E. coli* ribosome-binding site) and the protein fusion, and is unlikely to be caused by instability of the fusion protein as the Fcc_3 portion was kept to a minimal three residues on account of well-documented problems of attaching signal sequences to β -galactosidase (Slauch & Silhavy, 1991). Transcriptional readthrough from the upstream sequences is also unlikely as the promoter inserts were cloned downstream of the transcriptional terminator from the *rrnB* operon and indeed control plasmids with no inserts (EG322, EG323) gave activity levels of 7 Miller units aerobically and 5 Miller units anaerobically. We conclude, therefore, that *fccA* expression is regulated at the level of transcription and is regulated hierarchically in response to the redox potential of available terminal electron acceptors.

Promoter sequence requirements for regulation of *fccA*

As a means of determining the specific sequences required for *fccA* promoter function and the importance of the putative EtrA sites for *fccA* regulation, a series of promoter deletions was constructed and fused to the *lacZ* gene in a multicopy transcriptional fusion assay system (Parales & Harwood, 1993). The *fccA*-dependent *lacZ* expression levels of these fusions under anaerobic conditions were then assayed.

The constructs used in this assay are shown diagrammatically in Fig. 4. It is apparent from the relative promoter activities that deletion beyond position -167

Table 3. Expression of the *fccA-lacZ* transcriptional and translational fusions under different growth conditions

EG281, *fccA-lacZ* transcriptional fusion; EG282, *fccA-lacZ* translational fusion; EG322 (transcriptional control) and EG323 (translational control) have no insert. The numbers in parentheses are the factors by which the expression level was reduced compared to the maximal level observed (the level found in cells grown anaerobically with fumarate). Data are expressed in Miller units and were obtained from at least three independent experiments; values rarely differed by more than 10%.

Strain	Terminal electron acceptor					
	Oxygen	Fumarate	Nitrate	TMAO	Fumarate + TMAO	Fumarate + nitrate
EG281	75 (↓ 72)	5390	152 (↓ 35)	306 (↓ 18)	552 (↓ 10)	740 (↓ 7)
EG282	29 (↓ 78)	2265	69 (↓ 33)	124 (↓ 18)	222 (↓ 10)	358 (↓ 6)
EG322	7	5				
EG323	7	5				

		Plasmid	Aerobic	Anaerobic	Promoter activity %
K P H N D S E					
-623	← <i>orf1</i>	pEG210	800	9296	100
-414	← <i>lacZ</i>	pEG211	595	9603	103
-167	← <i>lacZ</i>	pEG644	595	7935	85
-103	← <i>lacZ</i>	pEG648	805	804	7
+49	← <i>lacZ</i>	pEG216	595	972	9
	← <i>lacZ</i>	pEG638	204	192	1
	<i>aac</i> → <i>lacZ</i>	pEG308	233	199	
	ΩSm → <i>lacZ</i>	pEG311	158	130	

Fig. 4. Deletion analysis of the *fccA* promoter. All fusions were cloned in pHRP309. Figures represent data (Miller units) from at least three independent experiments; values rarely differed by more than 10%. Percentage activities were calculated after correction for basal activity (the pHRP311 value). *aac*, confers gentamicin resistance. The extent of the DNA carried on each plasmid is numbered relative to the *fccA* transcription start site. K, *KpnI*; P, *PvuII*; H, *HpaI*; N, *NsiI*; D, *DraI*; S, *SnaBI*; E, *EcoRI*.

Table 4. Transcriptional activity of progressively deleted forms of the *fccA* promoter

The *fccA* promoter region was partially deleted using *Bal31* nuclease to yield derivatives with progressively shorter 5' non-coding sequences. These derivatives were transferred into pHRP309 and assayed in NCIMB 400 for β -galactosidase activity. Activities were measured at mid-exponential phase after growth either aerobically or anaerobically in medium supplemented with lactate and fumarate. Values shown are the means from at least three independent experiments. Individual values rarely varied by more than 10%.

	Deletion point relative to transcription start				
	-158	-154	-138	-124	-103
Anaerobic	8794	3870	2098	2622	804
Aerobic	643	645	642	677	805

(pEG644) had no effect on the regulation of *fccA* with respect to anaerobic growth on fumarate. The complementary deletion of DNA downstream from -167 (pEG638) confirmed that there were no elements upstream of this position (the *HpaI* restriction site) that

affected anaerobic regulation of *fccA* transcription. However, deletion of an additional 65 bp to -103 resulted in the loss of anaerobic inducibility.

To investigate this observation further, progressive deletions of the *fccA* promoter from -167 to -103 were constructed by the use of *Bal31* nuclease. The effects of these deletions on *fccA* transcription can be seen in Table 4. In relation to promoter sequence, the -158 deletion is up to the G base of the putative EtrA (FNR) box centred at -153.5 (Fig. 5). Removal of a further four nucleotides to -154 removes a half-site of this EtrA box, and the activity with this construct is 2.2-fold lower. Removal of a further 16 nucleotides removes all of this putative EtrA box and the resultant promoter activity is decreased by another twofold. Anaerobic inducibility of the promoter is lost with the deletion of a further 38 nucleotides.

Physiological function of flavocytochrome c_3

Construction of an *fccA* mutant. Previous reports (Myers & Myers, 1992; Tsapin *et al.*, 1995) suggested that *S. putrefaciens* possesses at least two fumarate reductases, raising the possibility that Fcc₃ may not be the terminal reductase for fumarate respiration and could have some

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-172  TGTAACTCGATGTGATCTATCTCCAATAAGCTATGCAAAATTGCATAT
          -158 ↓      -154 ↓      -138 ↓      -124 ↓
-122  ACCTCTTTATAACTATGCATGTTTGCATAGCGCATTTTATAGGCTTGTGAT
          ↓
          -105
-72   TGAGTTGTCTATTTCTGCCCTCTATTCTGGTTATCTGGTTGTTGATTGCGC
          -35
-22   CCCCTTAGGCAAGATTACATTGAGCGAAAATAATAGCTGATCAACTCGA
          -10      +1
+29   TGCTAATTTGGACCGTATTTTTAAAGTGAAGTGAAGTCAGTTTCACTTCA
          rbs      M K K M N L A V C
+79   GCCTAACTAAAACAATAAGAGGCTTCTATAGTTAATGAATAAACCGTCAT
+129  TACGTATAGAAGGGGGAGCAAAATGAAAAGATGAATCTTGCAGTCTGT

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Fig. 5. Sequence of the 5'-end of the *fccA* gene. The position of the transcription start site, as determined by primer extension, is indicated by the arrow at position +1 and the sequence is numbered relative to this location. Possible -10 and -35 sequences are underlined and the putative EtrA recognition sites are boxed. The putative ribosome-binding site (rbs) is indicated, just preceding the coding sequence which is shown as the protein translation.

Fig. 6. Immunochemical detection of Fcc₃. Cells were grown to mid-exponential phase and harvested, and approximately equivalent protein concentrations were loaded on each lane. Anaerobically grown cells were grown with lactate, fumarate and TMAO as detailed in the text. Lanes: 1, EG301 grown anaerobically; 2, EG301 grown aerobically; 3, NCIMB 400 grown anaerobically; 4, NCIMB 400 grown aerobically.

as yet undefined function. To confirm whether the *fccA* gene did indeed encode a respiratory fumarate reductase we constructed a strain of *S. putrefaciens* deleted for *fccA*. Using gene replacement we deleted virtually the entire *fccA* coding sequence as detailed in Methods. The DNA structure of the deletion was confirmed by Southern blot hybridization experiments with genomic DNA extracted from several putative mutant strains (data not shown). The absence of the Fcc₃ protein was confirmed in mutant strains by Western blotting of extracts from cells grown anaerobically in the presence of fumarate and TMAO (Fig. 6). Assays of total cell extracts of *S. putrefaciens* grown as above demonstrated a complete absence of methylviologen-dependent fumarate reductase activity, indicating that Fcc₃ is the sole fumarate reductase in *S. putrefaciens*.

Phenotype of an *fccA* mutant. Of the mutants obtained, one, EG301, was selected for further analysis. This strain was tested for the ability to grow on minimal media containing various carbon sources and electron acceptors. EG301 could grow aerobically with lactate as the sole source of carbon and energy. Anaerobically, no growth was apparent with fumarate as the sole terminal electron acceptor with either lactate or formate as the electron donor. The growth patterns of EG301 and wild-type *S. putrefaciens* using other terminal electron acceptors [nitrate, fumarate, TMAO, Mn(IV), sulphite and thiosulphate] were indistinguishable.

Complementation of an *fccA* mutant. To demonstrate that the phenotype of EG301 was indeed due to the disruption of the *fccA* gene and a consequent absence of the Fcc₃ protein and not to some as yet unidentified secondary effect we attempted to complement the $\Delta fccA$ mutation using various forms of the *fccA* gene (constructed as described in Methods) introduced into EG301 on the IPTG inducible expression vector pMM-B503EH (Michel *et al.*, 1995).

The ability of the expression constructs to complement the $\Delta fccA$ phenotype was assayed on minimal medium with fumarate as the sole terminal electron acceptor. EG301 carrying pEGX8, which encodes the complete mature Fcc₃ but without the secretory signal sequence, failed to grow on minimal medium with fumarate as the sole terminal electron acceptor whether or not IPTG was added. EG301 carrying pEGX1, which encodes the complete Fcc₃, did grow anaerobically with fumarate as the sole terminal electron acceptor but only when *fccA* expression was induced with IPTG.

Using site-directed mutagenesis, novel *fccA* gene constructs were made that enabled the flavin domain of Fcc₃ to be overproduced in two distinct forms, with and without a signal sequence. The construct carried on pEGX9 encoded the flavin domain only (residues 113–571 of the native protein) whereas pEGX11 encoded the flavin domain but onto which the Fcc₃ presequence had been added. This construct was made by deleting the DNA encoding the cytochrome domain (residues 5–99) from *fccA*. The production of these polypeptides was confirmed by Western blots of total cell protein from induced and non-induced cultures of EG301 carrying pEGX9 and pEGX11, respectively. When present in EG301, neither of these constructs supported anaerobic growth with fumarate as the sole terminal electron acceptor.

DISCUSSION

The soluble fumarate reductase flavocytochrome c_3 is the most abundant cytochrome in *S. putrefaciens* grown anaerobically with fumarate as the terminal electron acceptor. This enzyme behaves *in vitro* as a uni-directional fumarate reductase, suggesting that the physiological function of this enzyme is in fumarate respiration. It has, however, been suggested that *S. putrefaciens* may produce two or more fumarate reductases. Myers & Myers (1992) found fumarate reductase

activity in different subcellular fractions that they ascribed to periplasmic and cytoplasmic locations. Tsapin *et al.* (1995) identified EPR signals in whole cells of *S. putrefaciens* that were attributed to a fumarate reductase of the type found in the cytoplasmic membrane of *E. coli*. To investigate the physiological function of Fcc₃ we deleted the *fccA* structural gene from *S. putrefaciens* NCIMB 400 and examined the phenotypic consequences of the lack of Fcc₃ protein. The resulting strain, EG301, lacked detectable flavocytochrome c₃ protein and methylviologen-dependent fumarate reductase activity. Analysis of the phenotype of EG301 demonstrated that the cells could not grow anaerobically with fumarate as the terminal electron acceptor, but were unaffected in anaerobic growth with nitrate, TMAO, Mn(IV), thiosulphate and sulphite. We conclude, therefore, that Fcc₃ is required for fumarate respiration in *S. putrefaciens*, acting as the terminal reductase, and furthermore that Fcc₃ is the sole fumarate reductase in this organism. The nature of the interactions of Fcc₃ with other components of the *Shewanella* respiratory chain is unknown. It is not clear whether the immediate electron donor to Fcc₃ is soluble, like Fcc₃ itself, or membrane-bound. A candidate electron donor to Fcc₃ is the membrane-bound CymA protein (Myers & Myers, 1997). This is a tetrahaem c-type cytochrome with extensive similarity to NapC, which is thought to mediate electron transfer between quinones and the periplasmic nitrate reductase in organisms such as *Thiosphaera pantotropha* (Berks *et al.*, 1995). Mutations in the *cymA* gene result in a defect in growth with fumarate as well as other acceptors but there is no direct evidence for an interaction between CymA and Fcc₃.

The $\Delta fccA$ phenotype was complemented when Fcc₃ was supplied *in trans* on an expression vector, indicating that no other mutation was present in EG301 to account for the observed phenotype. The location of Fcc₃ is essential to the function of the enzyme as deletion of the signal sequence (pEGX8) from the protein resulted in a protein that remained in the cytosol and failed to complement the $\Delta fccA$ phenotype. Overproduction of the flavin domain of Fcc₃, with or without a signal sequence, did not allow EG301 to grow anaerobically with fumarate as the sole terminal electron acceptor.

To gain an insight into the regulation of the *fccA* gene its transcription was investigated by Northern blot hybridization. This analysis revealed a transcript of approximately 2 kb. This transcript is not of sufficient size to arise from the co-transcription of *fccA* and the following reading frame, *orf2*. Thus, *fccA* does not form part of an operon. The lack of a functional relationship between *orf2* and *fccA* is also indicated by the observation that disruption and partial deletion of *orf2* had no detectable effect on fumarate reduction or on anaerobic growth with a variety of acceptors (results not shown). Northern blot hybridization also revealed that *fccA* mRNA, like the Fcc₃ protein (Pealing *et al.*, 1992), was only detectable in *S. putrefaciens* grown anaerobically, suggesting that the gene is regulated at the level of transcription. The use of β -galactosidase operon and

protein fusions confirmed this to be the case. These reporter fusions also demonstrated that the *fccA* gene was regulated hierarchically with respect to the redox potential of available electron acceptors in the order of oxygen > nitrate > TMAO > fumarate.

The regulation of the *S. putrefaciens* fumarate reductase by TMAO contrasts with the situation in *E. coli*, where the presence of TMAO has no effect on *frdABCD* transcription (Jones & Gunsalus, 1985). It makes bioenergetic sense for *S. putrefaciens* to have evolved this regulatory control for the preferential use of TMAO ($E_m = +130$ mV) over fumarate ($E_m = +30$ mV) as not only is TMAO a considerably more electropositive terminal acceptor than fumarate but TMAO is also relatively abundant in the marine environment. It will be interesting to see whether this repression of *fccA* transcription is mediated by a homologue of TorR, the regulator of the *torCAD* operon which encodes the major TMAO reductase in *E. coli* (Méjean *et al.*, 1994), as sequences similar to the TorR box can be found in the promoter of *fccA*. It is known that the *tor* region is conserved in close relatives of *E. coli* (Jourlin *et al.*, 1995) but not whether the same is true in other gamma-proteobacteria such as *Shewanella*.

At the present time, the regulatory factors involved in *fccA* gene expression are unknown. Deletion analysis of the promoter region suggests that sequences between -158 and -103 relative to the transcription start site are vital to the regulation of the *fccA* gene. It is tempting to speculate that the decrease in promoter activity of these deletions is due to the removal of an EtrA box but the available evidence does not lend strong support to this view. The sequence in this region does not provide a convincingly good match to the consensus FNR-binding site and the distance between this region and the transcription start site is much greater than normally found. Abnormal spacing of FNR boxes has been reported in *Pseudomonas* G-179, where the FNR box for the *nirU* gene is 300 bp upstream of the transcript start (Ye *et al.*, 1993). Fumarate respiration is under the control of EtrA in *S. putrefaciens* (Saffarini & Nealson, 1993) but there is no evidence for a direct interaction between EtrA and the *fccA* promoter. Further biochemical and genetic work will be required to show whether EtrA and/or other proteins interact with the *fccA* promoter to mediate regulation in response to anaerobiosis and to the presence of nitrate and other electron acceptors.

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