Respiratory enzymes from *Shewanella* MR-1

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Abstract

Shewanella MR-1 is a Gram-negative, facultatively anaerobic bacterium isolated from Lake Oneida, New York. It can couple its anaerobic growth to the reduction of a wide variety of compunds such as nitrate, nitrite, TMAO, DMSO, fumarate, manganese(IV) and iron(III) oxides, sulfite and thiosulfate. Analysis of the genome sequence reveals the presence of a large number of respiratory enzymes. Three of these proteins were selected for further study: a decaheme cytochrome *c*, a heptaheme cytochrome *c* and a flavoprotein.

Decaheme 129 (Cyc129) is 37 % similar to MtrC, a decaheme protein from the same organism that has been shown to be involved in iron(III) and manganese(IV) respiration. The DNA sequence indicated the presence of a lipoprotein signal sequence but the protein is loosely associated to the membrane. Compared to the wild-type strain, no phenotypic differences were noted when the *cyc129* gene was disrupted by the insertion of an antibiotic cassette.

The second protein, heptaheme 202 (Cyc202) is a soluble, periplasmic protein and is the only heptaheme cytochrome *c* in *Shewanella* MR-1. Phenotypic studies indicate that it might be involved in the electron transport to the outer-membrane located iron-manganese reductases.

FccA56 is similar to the flavin domain of flavocytochrome c_3, the fumarate reductase from *Shewanella* MR-1. The gene encoding this protein is part of a cluster that also encodes a tetraheme *c*-type cytochrome and a histidine ammonia lyase-like protein. Substitution of the highly conserved amino acids involved in substrate binding suggests that fumarate is not the physiological substrate of FccA56, but has a similar substrate that contains only one carboxylate group. The protein was purified after overexpression in *E. coli*. A UV-visible absorption spectrum confirmed that the ~52 kDa protein has absorption maxima at 450 and 380 nm, characteristic for flavoproteins. Redox titrations using absorbance changes at 450 nm gave midpoint potentials of -212 and -190 mV. Kinetic studies showed that, with the exception of fumarate, no reductase activity could

be detected with any of the compounds tested. The low turnover (0.15 s^{-1}) and high value of Km for fumarate (220 μM) indicate that this is not a true substrate. A null mutant was constructed but no phenotypic defect has been identified to date.

Abbreviations

Amp	ampicillin
AMPS	amonium persulfate
bp	base pair(s)
BSA	bovine serum albumin
°C	degree Celsius
Cml	chloramphenicol
Cyc	cytochrome c
Da	Dalton
DCIP	dichloroindophenol
ddNTP	dideoxynucleoside triphospate
DEAE	diethylaminoethyl
DIG	digoxigenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	diaminoethanetetraacetic acid
FAD	flavin adenine dinucleotide
Fcc3	flavocytochrome c_3
Frd	fumarate reductase
FNR	fumarate-nitrate reduction
Gm	gentamicin
h	hour
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Km	kanamycin

l	litre
LB	Luria-Broth medium
M	molar
min	minute
nm	nanometre
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppm	parts per million
PMSF	phenylmethylsulphonyl fluoride
Rf	rifampicin
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Sm	streptomycin
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N', N' tetramethylethylenediamine
TMAO	trimethylamine N-oxide
TMBZ	tetramethylbenzidine
Tris	2-amino-2(hydroxymethyl)-1,2-propandiol
UV	ultra violet
v/v	volume per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
w/v	weight per volume

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Chapter I Introduction

1.1 *Shewanella*- the most versatile bacteria

Shewanella spp are facultatively anaerobic Gram-negative motile rods (0.8 by 1.6 µm; Fig 1.1) isolated from a variety of habitats: fresh water- *S. putrefaciens* MR-1 (Myers & Nealson, 1990); sea water- *S. frigidimarina* NCIMB400 (Lee *et al.*, 1977), sea ice- *S. gelidimarina* (Nichols *et al.*, 2000), crude oil pipelines- *S. putrefaciens* 200 (Obuekwe & Westlake, 1980), associated with eastern oyster- *S. colwelliana* (Abu *et al.*, 1994) or red algae- *S. alga* (Simidu *et al.*, 1990), anaerobic sandstones- *S. putrefaciens* CN32 (Wildung *et al.*, 2000), deep-sea sediments: *S. benthica* and *S. violacea* (Yamada *et al.*, 2000). *Shewanella* is an opportunistic pathogen and most human isolates occur as part of a mixed bacterial flora (Khashe & Janda, 1998). A number of monomicrobic illnesses due to *S. putrefaciens* have been documented and include chronic leg ulcers (Dominguez *et al.*, 1996), abscesses (Yoke *et al.*, 1997), septicaemia (Kim *et al.*, 1989) and infective endocarditis (Dhawan *et al*, 1998). Recent 16S rRNA sequencing and study of the whole-cell protein profile have shown that 84 % of human isolates reside within the *S. alga* group (Khashe & Janda, 1998).

The ability of *Shewanella* spp to adapt to so many and different habitats is supported by a complex respiratory pathway that allows them to metabolise a wide range of substrates. Growth can be coupled to the reduction of different electron acceptors, from highly electropositive compounds (oxygen) to very electronegative acceptors (e.g sulfite; see Table 1.1). To date little is known about the anaerobic electron transport chain. Fumarate reductases have been isolated from *S. frigidimarina* NCIMB400

(Pealing *et al.*, 1992), and *S. putrefaciens* MR-1 (Myers & Myers, 1992) and catalyse, essentially irreversibly, the reduction of fumarate to succinate (see Chapter 1.3.2.2).

Fig 1.1 *Shewanella putrefaciens* MR-1. Scanning electron microscopy was carried out by Chris Jeffree in the "Electron microscopy lab" University of Edinburgh

Fumarate reductase expression is induced by fumarate and regulated by EtrA (Saffarini & Nealson, 1993), a fumarate-nitrate respiration regulator (see Chapter 1.4).

TMAO reductase has also been isolated from *S. frigidimarina* NCIMB400 (Clarke & Ward, 1988) and *S. massilia* (Dos Santos *et al.*, 1998). The enzyme is inducible at low oxygen concentrations even when the substrate is absent, probably as part of a strategy to cope with anaerobiosis (Easter *et al.*, 1983; see Chapter 1.3.2.3).

Due to its ability to reduce nitrate to dinitrogen and nitrous oxide, *Shewanella* is considered a denitrifier (Samuelsson *et al.*, 1988). Anaerobic growth with nitrate led to rapid and complete reduction of nitrate to nitrite and the formation of nitrous oxide. Based on hybridisation of *Shewanella* MR-1 chromosomal DNA with different nitrite reductase genes, it was deduced that nitrite reductase in this bacterium is a heme *c* and

heme d_1 containing enzyme (Krause & Nealson, 1997). A partially purified membrane-bound nitrite reductase and a three-subunit nitrate reductase were isolated (Krause & Nealson, 1997) which support the presence of a denitrification system.

Shewanella exhibits an unusual feature, common to only a few other bacteria like *Geobacter metallireducens* and *Desulfovibrio* spp, which is to metabolise insoluble iron and manganese oxides (Myers & Nealson, 1988). A small tetraheme cytochrome which is involved in Fe(III) reduction was isolated from *S. frigidimarina* NCIMB400 (Gordon *et al.*, 2000), but no specific manganese reductase has been found. MtrB, a decaheme protein from *S. putrefaciens* MR-1, is the only protein isolated so far that is required for both iron and manganese reduction (Beliaev & Saffarini, 1998). However, another two decaheme proteins OmcA and MtrC were identified in the same strain and disruption of their coding sequences decreased the ability of the strain to reduce manganese by 45 and 75 % respectively (Myers & Myers, 2001). The ability to metabolise insoluble oxides might be explained by the presence of multiheme *c*-type cytochromes on the outer membrane (Myers & Myers, 1992b) which allows physical contact between the bacterium and the oxides and together with tetraheme proteins might form an electron wire between the inner and the outer membrane (Myers & Myers, 1997).

Significant metal discharge from industrial plants into the environment occurs and *Shewanella* could be used in bioremediation of contaminated waters and waste streams by converting the highly soluble compounds to insoluble, less bioavailable products. It can reduce uranium U(VI) to U(IV) by using a membrane associated multicomponent enzyme system (Wade & Di Christina, 2000), chromium Cr(VI) to

Cr(III) by using a cytoplasmic membrane bound enzyme (Myers *et al.*, 2000) and technetium (VII) to insoluble products in lower oxidation states (Wildung *et al.*, 2000).

The sequencing of the *S. putrefaciens* MR-1 genome is currently in progress and preliminary analysis indicates the presence of a large number of *c*-type cytochromes and other respiratory enzymes (Chapter III), which correlates with the extreme versatility of this Gram negative bacterium. Analysis of these putative proteins and their comparison with known enzymes can supply extra information on the protein family to which they might belong, their role and probable location in the cell and different aerobic or anaerobic electron transport chains. Three of these proteins were further studied: a decaheme, probably outer membrane-bound, a unique heptaheme protein (both *c*-type cytochromes) and a flavoprotein that resembles the flavin domain of flavocytochrome c_3, a soluble fumarate reductase.

1.2 Bacterial electron carriers

Most bacteria obtain their energy from the oxidation of organic and inorganic substrates with the aid of diverse electron transport chains, which include flavoproteins, iron-sulfur proteins, quinones, cytochromes and other redox components.

1.2.1 Flavoproteins

Flavoproteins are electron carriers that have FMN or FAD as prosthetic group (see Fig 1.2).

Because of their chemical versatility, flavins are involved in a variety of biological processes. Flavoproteins have been discovered as playing signal transduction

roles in programmed cell death (Susin *et al.,* 1999); they are involved in soil detoxification of aromatic pollutants (Dagley, 1987), in several light-dependent processes, such as photosynthesis, light-dependent repair of DNA damage and the photoreduction of DNA dimers (Jorns *et al.*, 1987) and in plant phototropism, as part of the blue-light receptors (Briggs *et al.*, 1999).

X = H → riboflavin
X = PO_3H_2 → FMN
X = ADP → FAD

oxidised flavin ⟷ semiquinone ⟷ fully reduced flavin

Fig 1.2 The structure and redox states of flavins

The redox chemistry of the flavin is restricted to the isoalloxazine ring. The ribityl chain is not involved in catalysis, but serves to anchor the coenzyme at the active site. Reduction of the flavin occurs reversibly by two one-electron steps or one two-

electron step, involving changes which affect N(1), C(4a) and N(5) (Ghisla & Massey, 1986).

Flavoproteins differ in their redox potentials and which reaction they catalyse. These differences are due to the protein environment and not to the flavin itself. If the original flavin can be removed under conditions that avoid denaturing the protein, most flavoproteins will accept into their active sites chemically modified flavin ring structures, if the N(10) side chain is the appropriate one. A large number of chemically modified flavins have been synthesised with different substituents at various positions all around the isoalloxazine ring system (Ghisla & Massey, 1986).

1.2.2 Quinones

Quinones are lipid-soluble molecules that mediate electron transport between respiratory proteins. They contain isoprenoid chains that contribute to their lipid solubility in the cytoplasmic membrane bilayer. There are three quinol species: ubiquinone (Q), menaquinone (MQ) and demethylmenaquinone (DMK; see Fig 1.3). The number of isoprene units varies but is typically between 4 and 10, and is 8 for *E. coli.*

From mutagenesis studies in *E. coli*, a general pattern has emerged: Q is used for oxygen respiration, Q and DMK are used for nitrate respiration, and MK and DMK are used for anaerobic respiration with acceptors other than nitrate (Wissenbach *et al.*, 1992). Aerated cells contain about 4 to 5 fold more Q than MK+DMK, whereas anaerobic cells contain about one third as much Q as MK+DMK (Wissenbach *et al.*, 1990).

a) ubiquinone

d) ubiquinol

b) menaquinone

e) menaquinol

c) dimethylmenaquinone

f) dimethylmenaquinol

Fig 1.3 The structure of quinones

a, b, c - oxidised quinones

d, e, f - reduced quinones

The value of n can be 4-10 and is 8 for *E. coli*

1.2.3 Cytochromes

Cytochromes are electron carriers that have heme as the prosthetic group. Hemes are categorised on the basis of the side chains attached to the pyrrole rings. In the center of each heme there is an iron atom which is bound to the nitrogen of the pyrrole rings (Fig 1.4). The iron is the electron carrier and is oxidised to the ferric or reduced to the ferrous form during electron transfer.

Heme containing proteins display a diverse range of biological functions: electron transfer reactions (catalysed by *b*- and *c*-type cytochromes), oxygen transport and storage (hemoglobin, myoglobin), oxygen reduction to the level of water (cytochrome oxidase), oxygenations of organic substrates (cytochrome P450) and reduction of peroxides (catalase and peroxidase). This range of activities can be extended by combining heme groups with other cofactors (flavins) and metal ions (molybdenum, copper).

Hemes that are in respiratory components are hemes *b,c,d* and *o*. Heme *b* (protoheme IX; Fig 1.4) forms the prosthetic group in different enzymes like *b*-type cytochromes and cytochrome P450, hemoglobin and myohemoglobin, catalase and most peroxidases.

Fig 1.4 Heme *b* (protoheme IX)

Fig 1.5 Heme *c*

Fig 1.6 Heme *d*

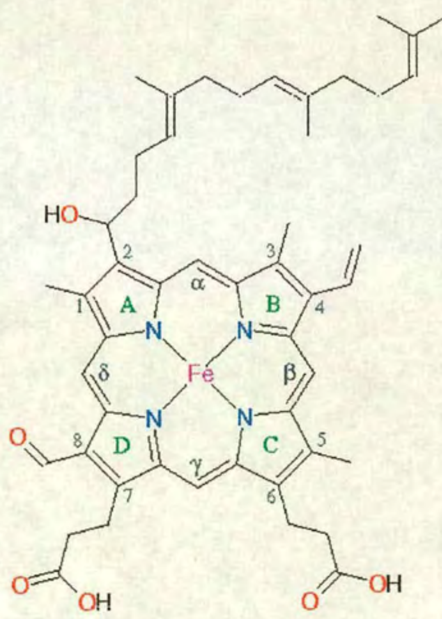

Fig 1.7 Heme *a*

Heme *c* (Fig 1.5) differs from heme *b* by being covalently bound to the protein by two thioether linkages between the heme vinyl groups and two cysteines. These occur

in a characteristic Cys-X-X-Cys-His motif. The *c*-type cytochromes will be discussed in more detail in Chapter III.

Hemes *d* and *o* are associated with one of the terminal oxidases in prokaryotes. Heme *d* (Fig 1.6) is found as a prosthetic group in cytochrome *bd*, present at low levels when *E. coli* is grown with high aeration, but is maximally present under microaerophilic conditions. Heme *o* is the biosynthetic precursor of heme *a* (Fig 1.7; Svensson *et al.,* 1993) and is present in respiratory oxidases of many bacteria. For example cytochrome aa_3 of cytochrome oxidase, in which one *a*-type heme acts as a simple electron transfer agent and heme a_3 provides a binuclear site along with Cu_B at which oxygen reduction takes place (Malatesta *et al.,*1995).

1.2.4 Iron-sulfur proteins

Iron-sulfur proteins are present in a number of respiratory proteins like the bc_1 complex, NADH dehydrogenase, succinate dehydrogenase and TMAO reductase. They contain covalently bound non-heme iron and acid labile sulfur (when the pH is lowered to approximately 1, H_2S is released from these proteins). Generally, a protein contains clusters in which the iron and the sulfur are present in a 1:1 ratio. There may be more than one cluster per protein. The electron may be highly delocalised and the entire cluster is thought of as carrying one electron, regardless of the number of iron atoms.

The Fe-S clusters are located within a protein by covalent bonds between the iron molecule and specific cysteine residues in the polypeptide chain. There are bi-, tri- and tetranuclear clusters. Binuclear [2Fe-2S] clusters (Fig 1.8) consist of two atoms of both iron and sulfur, with each iron additionally bonded to one or two cysteine residues.

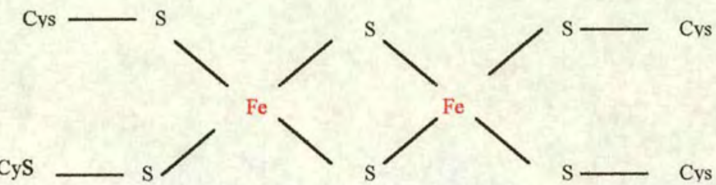

Fig 1.8 Schematic representation of a [2Fe-2S] binuclear cluster

[4Fe-4S] is a tetranuclear cluster where four atoms of iron and four of sulfur are bound in a cubane pattern (Fig 1.9), with each iron additionally bound to a single cysteine residue.

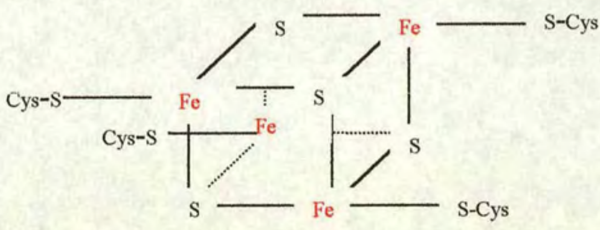

Fig 1.9 Schematic representation of a [4Fe-4S] cluster

A [3Fe-4S] cluster has a similar structure to [4Fe-4S], except that one of the cysteine residues and one of the iron atoms are not present.

Iron-sulfur proteins can have diverse functions. Many are involved in electron transport as components of the electron transfer system. Some Fe-S clusters are constituents of enzyme-active sites but are not involved in oxidation-reduction processes, for example aconitase (Beinert, 1990). In other cases the Fe-S center may

have a strictly structural role, as in *E. coli* endonuclease III (Kuo *et al.,* 1992). Some proteins containing an iron-sulfur centre are also redox sensing regulatory proteins (FNR and FNR-homologues; Khoroshilova *et al.*, 1995).

1.3 Respiration

Bacteria often encounter changes in their environment, including fluctuations in the levels of external oxygen. Unlike strict aerobes or strict anaerobes, which can survive only either in the presence or absence of oxygen, facultative anaerobes can cope with changes in environmental oxygen levels by sensing oxygen concentration and shifting cellular metabolism accordingly. The changes in metabolism in response to changes in oxygen availability include adjustments to the route and rate of carbon source use and the pathways of electron flow to maintain an oxidation-reduction balance. Different components can be substituted in the membrane in place of or in addition to other components as they are needed and can be fully functional parts of the entire system. There are three types of respiratory components:

1. substrate specific dehydrogenases, which carry out the oxidation of organic substrates and feed electrons into the quinone pool
2. quinones, which deliver reducing equivalents to the terminal oxidoreductases
3. terminal oxidoreductases which reduce the terminal electron acceptors.

Table 1.1 Midpoint potentials of electron donor and acceptor couples

Couple	Potential Eo(mV)
½ O_2/H_2O	+818
Fe^{3+}/Fe^{2+}	+771
NO_3^-/NO_2^-	+433
NO_3^-/NH_4^+	+360
½ $S_4O_6^-/S_2O_3^{2-}$	+170 to +24
DMSO/DMS	+160
TMAO/TMA	+130
ubiquinone/ubiquinol	+113
fumarate/succinate	+30
$FMN/FMNH_2$	-190
pyruvate/lactate	-190
$FAD/FADH_2$	-220
$NAD^+/NADH$	-320
$S_2O_3^{2-}/S^{2-}+HSO_3^-$	-402
H^+/ ½ H_2	-414
CO_2/HCO^-	-432

There is a hierarchy of electron acceptors that are used, oxygen being the most preferred, followed by nitrate (see Table 1.1). For example, in the presence of oxygen, genes for nitrate reductase, fumarate reductase and other reductases are repressed and only aerobic respiration can take place. In the absence of oxygen but in the presence of nitrate, nitrate reductase genes are induced, but the genes for fumarate reductase are

repressed. The reason that nitrate is used preferentially during anaerobiosis is that it has a more positive redox potential than the alternative electron acceptors and more energy is available from electron transport.

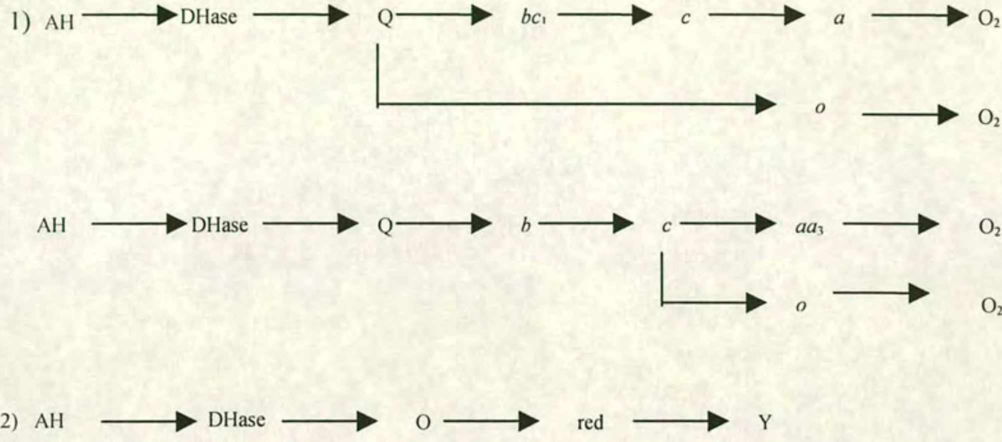

Fig 1.10 Generalized electron transport pathways found in bacteria

1. Aerobic respiration: a dehydrogenase (DH-ase) complex removes electrons from an electron donor and transfers these to a quinone. The electrons are transferred to an oxidase complex via a branched pathway. Depending upon the bacteria, the pathway may branch at the quinone (Q) or at the cytochrome. Many bacteria have cytochrome bc_1, cytochrome *c* and cytochrome aa_3 in one of the branches and in this way may resemble mitochondria. Others do not have a bc_1 complex and may not have cytochrome aa_3.

2. Anaerobic respiration: under anaerobic conditions, electrons are transferred to reductase complexes which are synthesised anaerobically. Several reductases exist, each one specific for the electron acceptor.

Y represents either an inorganic electron acceptor other than oxygen or an organic electron acceptor.

The routes to the terminal electron acceptors in bacteria are branched, the branch point being at the quinone or cytochrome. The ability to synthesise branched electron transport pathways to oxygen confers flexibility in bacteria; this can be seen with bacteria that can grow under either aerobic or anaerobic conditions (Fig 1.10).

1.3.1 Aerobic respiration and terminal oxidases

When grown aerobically, *E. coli* produces two different membrane-bound cytochrome oxidase complexes, cytochrome bo_3 (cytochrome *o*) and cytochrome *bd* (cytochrome *d*), resulting in a branched respiratory chain to oxygen that does not involve the cytochrome bc_1 complex or cytochrome *c* oxidase (Fig 1.11).

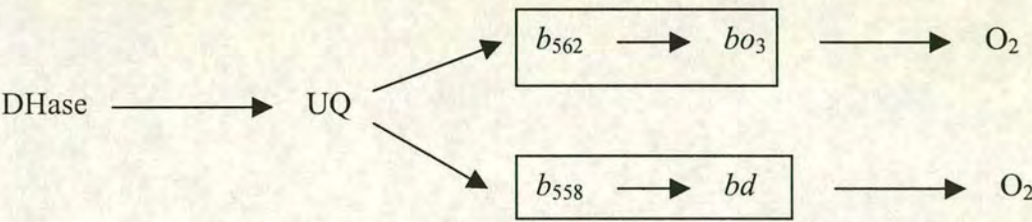

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Fig 1.11 Aerobic respiratory chain in *E. coli*

Both enzymes (cytochrome bo_3 and cytochrome *bd*) are quinol oxidases that form water as the product of oxygen reduction. Cytochrome bo_3 has homologues in all aerobic bacteria as well as in eukaryotes (mitochondrial cytochrome *c* oxidase). Expression of *E. coli* oxidases is regulated by the Arc and FNR systems (see Chapter 1.4; Cotter & Gunsalus, 1990). Decrease in the oxygen concentration results in the induction of cytochrome *bd* (Bogachev *et al.,* 1993). An increase in the growth medium temperature was shown to induce accumulation of cytochrome *bd* specific mRNA in *E.*

coli (Wall *et al.,* 1992). It was speculated that the role of cytochrome *bd* might be to scavenge oxygen and inhibit the degradation of oxygen sensitive enzymes present under anaerobic or microaerophilic growth conditions (Hill *et al.,* 1990).

Paracoccus denitrificans differs from *E. coli* in that it has a bc_1 complex and a cytochrome aa_3 oxidase (cytochrome *c* oxidase) in addition to a cytochrome bo_3 pathway.

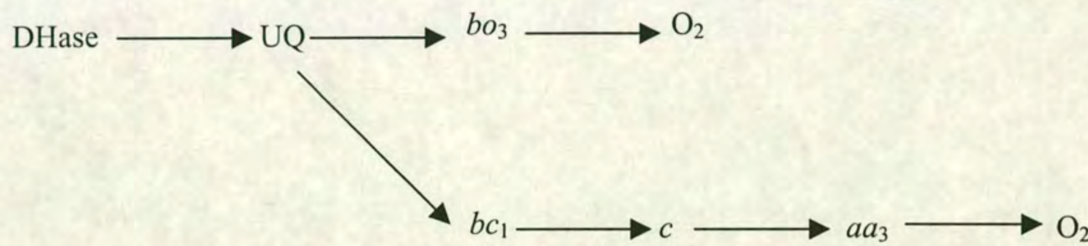


Fig 1.12 Aerobic electron transport in *P. denitrificans*

Cytochrome bc_1 complex was initially isolated as a "supercomplex" along with cytochrome *c* oxidase and the membrane bound cytochrome c_{552} (Berry, 1985). The bacterial cytochrome aa_3 is a three subunit enzyme in *P. denitrificans*. These three subunits have eukaryotic equivalents encoded by mitochondria. The bovine mitochondrial holoenzyme has an additional ten subunits which are encoded in the nucleus (Capaldi, 1990). Subunit I contains heme *a* and heme a_3 prosthetic groups, which together with Cu_B forms the active site. Subunit II contains a Cu_A center.

Rhodobacter sphaeroides has three distinct respiratory oxidases, two of which can utilize cytochrome *c* as a substrate (Garcia-Horsmann *et al.,*1994) and one which is quinol oxidase.

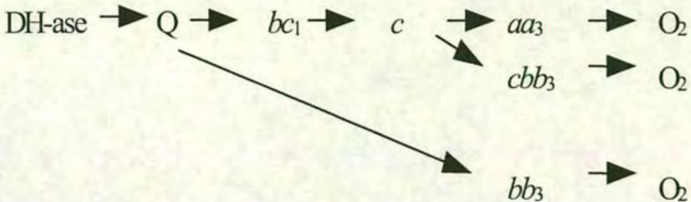


Fig 1.13 Aerobic respiration in *R. sphaeroides*

1.3.2 Anaerobic respiration and terminal reductases

1.3.2.1 Nitrate reductases

Nitrate is the preferred terminal electron acceptor when oxygen is absent, because of its high midpoint redox potential (E=+430 mV; see Table 1.1). Nitrate reduction occurs in bacteria only under reduced oxygen tension and the end products are excreted back into the environment.

E. coli possesses three nitrate reductases: A, Z, and Nap.

A. Nitrate reductase A is a membrane-bound enzyme encoded by the *narGHJI* operon (Sodergren *et al.,* 1988) and is similar to the one isolated from *P. denitrificans*, which couples the oxidation of physiological substrates to the reduction of nitrate and generation of a proton gradient. The active site of the enzyme is contained in the NarG subunit (α subunit), which faces the cytoplasm and coordinates a molybdopterin guanine dinucleotide cofactor (Chaudhry, 1983). NarH (β subunit) binds three [4Fe-4S] centres and one [3Fe-4S] centre (Berks *et al.,*1995). NarI (γ subunit) is a transmembrane subunit which anchors the α and β subunits to the membrane (Fig 1.14).

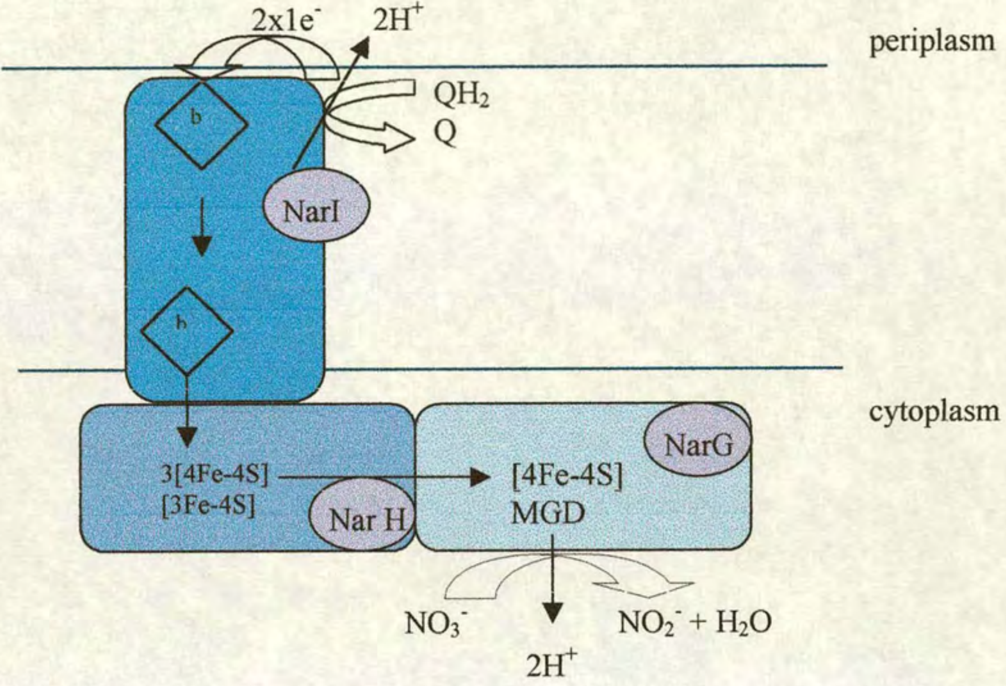


Fig 1.14 Subunit and cofactor arrangement in the membrane-bound nitrate reductase (Berks *et al.*, 1995)

NarJ is required in the assembly of the physiologically active nitrate reductase (Dubourdieu *et al.*, 1992) but although it is encoded by the *narGHJI* operon, it is not found in the purified enzyme. Upstream of the operon there is a fifth gene, *narK*, which encodes a protein that enhances nitrate uptake or nitrite excretion (Rowe *et al.*, 1994). It is likely that NarK prevents the intracellular concentration of nitrite from rising to toxic levels under conditions of anaerobic nitrate respiration (Rowe *et al.*, 1994).

Expression of nitrate reductase A is activated by the phosphorylated NarL protein (NarL~P; see Chapter 1.4) during anaerobic growth in the presence of nitrate

(Rabin & Stuart, 1993). When the enzyme is inactivated in *P. denitrificans* by transposon insertion, the mutant strain retains wild-type levels of nitrate reductase when grown anaerobically because of the periplasmic nitrate reductase (Bell *et al.,* 1993).

B. Unlike *P. denitrificans, E. coli* possesses a second membrane-bound nitrate reductase, nitrate reductase Z, encoded by the *narZYWV* operon. It has similar biochemical properties to nitrate reductase A but is expressed at a very low level and is unaffected by anaerobiosis or the presence or absence of nitrate (Blasco *et al.,*1990).

C. The third enzyme is a periplasmic nitrate reductase, which in *E. coli* is encoded by *napFDAGHBC* and resembles the soluble nitrate reductase from *P. denitrificans*. NapA is the nitrate reductase and contains a molybdenum cofactor (Benett *et al.,* 1994) and a [4Fe-4S] centre; NapB binds two *c*-type heme groups (cytochrome c_{552}). NapC consists of a periplasmic domain containing four *c*-type heme groups and an N-terminal membrane spanning region and is the direct electron donor to the NapAB complex (Berks *et al.,* 1995). The function of NapD is unknown. It is predicted to have a cytoplasmic location but lacks conserved amino acids likely to bind redox cofactors. As with NapD, NapE might be involved in the interaction between NapC and quinol oxidase (Berks *et al.,* 1995). Although NapF, NapG and NapH all contribute to the maximal rate of nitrate reduction in the periplasm, their roles remain unknown (Potter *et al.,* 1999).

All of the *nap* operon sequences determined so far include four similar genes in the same order, *DABC*, but additional genes are always present. The *nap* operon in *R.*

sphaeroides consists of 7 genes in the order *napKEFDABC* (Reyes *et al.,* 1998). NapK has not been found in other bacteria and the function of its product is unknown.

Transcription is induced by the FNR protein (see Chapter 1.4) during anaerobic growth (Grove *et al.,* 1996). Transcription is also induced by NarL and NarP in response to nitrate (Rabin & Stuart, 1993).

P. denitrificans can grow anaerobically using nitrate as an electron acceptor, reducing it to nitrogen gas in a processes called denitrification catalysed by nitrate reductase, nitrite reductase, nitrous oxide reductase and nitric oxide reductase. The electron transport pathway includes several branches to the individual reductases (Fig 1.15).

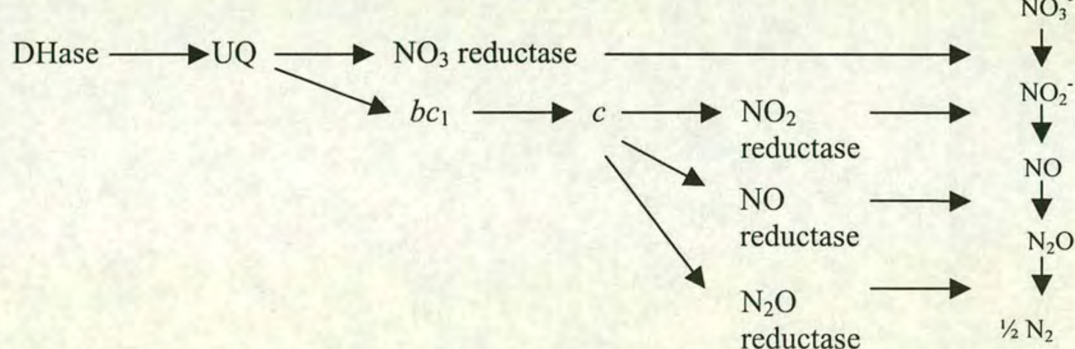


Fig 1.15 Anaerobic electron transport in *P. denitrificans* when bacteria are grown anaerobically using nitrate as the electron acceptor; the cytochrome aa_3 levels are very low and the electrons travel from ubiquinone to nitrate reductase and also through the bc_1 complex to nitrite-, nitric oxide- and nitrous oxide reductase (Grove *et al*, 1996).

S. putrefaciens MR-1 is capable of reducing nitrate to ammonia (Samuelsson *et al.*, 1985). Anaerobic growth with nitrate leads (see Chapter 1.1) to rapid and complete

reduction of the nitrate to nitrite and the formation of nitrous oxide. Oxygen inhibits nitrate respiration but it does not completely suppress the nitrate respiration system (Krause & Nealson, 1997).

Certain bacteria (strict aerobes) can carry out assimilatory nitrate reduction, but cannot use nitrate as an electron acceptor for anaerobic growth. Other bacteria carry out both assimilatory and respiratory nitrate reduction. Assimilatory nitrate reductases are soluble enzymes that are induced by nitrate and repressed by ammonia. In *B. subtilis*, the genes encoding the subunits of assimilatory nitrate (*nasB* and *nasC*) and nitrite (*nasD* and *nasE*) reductases constitute an operon together with the *nasF* gene, which is involved in the formation of siroheme, the nitrite reductase cofactor, and *nasA* which encodes a nitrate transporter (Glosser *et al.,* 1995).

1.3.2.2 Fumarate reductase

Fumarate reductase is induced anaerobically by fumarate but repressed by oxygen or anaerobically by nitrate. Anaerobic induction is dependent on FNR or FNR-like proteins for activation (see Chapter 1.4)

Fumarate reductase (Frd) along with succinate dehydrogenase (Sdh) form a family of highly conserved respiratory proteins. They are usually composed of four non-identical subunits: a large flavoprotein to which FAD is covalently attached (FrdA), a Fe-S protein (FrdB) and two smaller hydrophobic membrane subunits (FrdC and FrdD). The covalent attachment of FAD to the flavoprotein is a highly conserved feature in this enzyme family (Robinson *et al.*, 1995). Mutational alteration of the histidine residue that anchors the FAD in *E. coli* Frd resulted in an enzyme that contains noncovalently bound

FAD, retains the fumarate reductase activity, but has lost the succinate dehydrogenase activity (Blaut *et al.,* 1989).

E. coli fumarate reductase and succinate dehydrogenase exhibit substantial similarity in amino acid sequence, cofactor composition and mechanism. Mutations in Frd or Sdh complexes can decrease growth under appropriate conditions (Schröder *et al.*, 1991). Frd is an excellent succinate dehydrogenase and catalyses succinate oxidation at 30-40 % of the rate at which it reduces fumarate (Blaut *et al.*, 1989).

The FrdB subunit contains three Fe-S centres: [2Fe-2S] (1), [4Fe-4S] (2) and [3Fe-4S] (3) (Iverson et al, 1999). Centre 2 requires the presence of the FrdA subunit for proper assembly (Johnson *et al.*, 1988). Centre 3 is essential for electron transport as well as proper assembly of the Frd complex (van Hellemond & Tielens, 1994). Systematic mutation of the cysteine residues that anchor the Fe-S centres showed that the clusters are arranged in a separate domain within the subunit (Guigliarella *et al.*, 1996).

W. succinogenes fumarate reductase resembles that of *E. coli* but contains only one hydrophobic subunit with two *b*-type heme groups. FrdB consists of two domains: an N-terminal "plant ferredoxin" domain which binds the [2Fe-2S] centre and a C-terminal "bacterial ferredoxin" domain, which binds [4Fe-4S] and [3Fe-4S] clusters (Lancaster *et al.*, 1999).

Related to *Wolinella* FrdA is the protein isolated from *Helicobacter pylori* (80 % sequence identity; Alm *et al.*, 1999). Disruption of *frdA* abolishes fumarate reductase activity (Ge *et al.*, 1997) and inhibitors of fumarate reductase have bactericidal effects

on the viability of *H. pylori in vitro*, suggesting that FrdA could be a potential therapeutic target (Ge *et al.*, 2000).

There are organisms including *S. cerevisiae* (Arikawa *et al.*, 1998) and *Shewanella* spp (Pealing *et al.*, 1992) that possess a soluble fumarate reductase which catalyses, essentially irreversibly the reduction of fumarate to succinate. The *Shewanella* spp mature protein contains three domains: an N-terminal tetraheme *c*-type cytochrome, a larger C-terminal flavoprotein domain which contains noncovalently-bound FAD at the active site and a clamp domain which is likely to be involved in controlling access of substrate to the active site (Taylor *et al.,* 1999). The *fccA* disruption in *S. frigidimarina* NCIMB400 completely abolishes fumarate reduction, but anaerobic growth with nitrate, TMAO, Mn (IV), thiosulfate and sulfite remain unaffected (Gordon *et al.*, 1998). However, a second fumarate reductase-like protein with unknown function and induced only during respiration with soluble Fe (III) has also been isolated (Dobbin *et al.*, 1999).

1.3.2.3 TMAO/DMSO reductase

TMAO is widely distributed in tissues of marine fish and invertebrates where it acts as an osmoprotector. In decaying organisms it can be reduced to volatile TMA by a variety of bacteria including enteric bacteria, marine bacteria (*Shewanella* and *Vibrio* spp) and photosynthetic bacteria found in ponds (*Rhodobacter* spp; Barett & Kwan, 1985). Although these strains are only distantly related, the TMAO reductase systems are related (Dos Santos *et al.,* 1998).

In *E. coli*, TMAO reductase allows anaerobic growth on non-fermentative carbon sources. Although it is only expressed in anaerobiosis, it escapes control by the FNR

protein (see Chapter 1.4; Spiro & Guest, 1990). Even though the redox potential of the TMAO/TMA couple is significantly lower than that of the nitrate/nitrite couple (see Table 1.1), the expression of the TMAO system is not hierarchically repressed by nitrate (Silvestro *et al.*, 1988). In *Shewanella massilia*, the enzyme is encoded by the *torCAD* operon whose expression is induced in the presence of TMAO (Mejean *et al.*, 1994); it comprises a periplasmic TMAO reductase (TorA; Dos Santos *et al.*, 1998) which requires a molybdenum cofactor at the active site (Czjzek et al, 1998) and a membrane-bound pentaheme *c*-type cytochrome (TorC) which is involved in electron transfer to TorA (Mejean *et al.*, 1994). TorA shares high similarity with both the *E. coli* TorA and *Rhodobacter* DorA periplasmic proteins (Dos Santos *et al.*, 1998). The *torD* gene encodes a cytoplasmic TorA chaperone (Pommiere *et al.*, 1998).

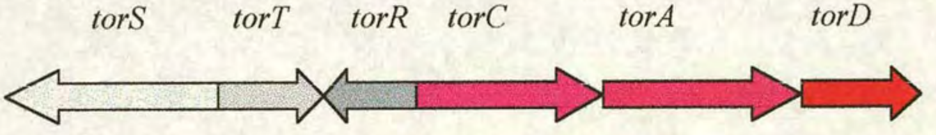


Fig 1.16 Physical map of the *tor* locus in *E. coli*

In addition to the *torCAD* operon, the *E. coli tor* locus contains three regulatory genes (Fig 1.16), *torR, torS and torT* (Jourlin *et al.*, 1996). These regulatory genes encode positive regulators essential for the *torCAD* operon expression. TorS and TorR form a regulatory system in which TorS detects the presence of TMAO in the medium. TorT is a periplasmic protein similar to the periplasmic ribose-binding protein (Ansaldi *et al.*, 1999).

The TMAO reductases from *E. coli, S. putrefaciens* and *Roseobacter denitrificans* are unable to reduce S-oxide compounds such as DMSO. DMSO reductase from *E. coli, Rhodobacter capsulatus* and *Proteus vulgaris* can reduce TMAO as well as other N- and S-oxides (Dos Santos *et al.,* 1998). An attractive hypothesis is that these systems have evolved from a common ancestor capable of reducing a broad range of substrates. Since bacterial species containing these related respiratory systems are distant from each other, it is probable that such bacteria have acquired the TMAO/DMSO reductase function by horizontal gene transfer (Dos Santos *et al.,* 1998).

In *E.coli* the *dmsABC* operon is closely related to the Tor system and is expressed regardless of the substrate during anaerobiosis. It encodes the three subunits of the DMSO reductase complex: DmsA is the molybdenum-containing subunit, DmsB the electron transfer subunit, which contains four [4Fe-4S] clusters, and DmsC is the membrane anchor (Sambasivarao *et al.,* 2000).

1.4 Fermentation

In the absence of external electron acceptors, some bacteria can grow by fermentation, where energy is generated by substrate level phosphorylation and pyruvate is used to reoxidise NADH, allowing glycolysis to continue. Because a respiratory electron transfer chain linked to terminal electron acceptors is absent in fermentation, recycling of NADH is accomplished by conversion of pyruvate to fermentation products.

B. subtilis has a very inefficient glucose fermentation pathway; it can grow by fermentation either when both glucose and pyruvate (Fig 1.17) are provided or when glucose and mixtures of amino acids are present (Nakano *et al.*, 1997).

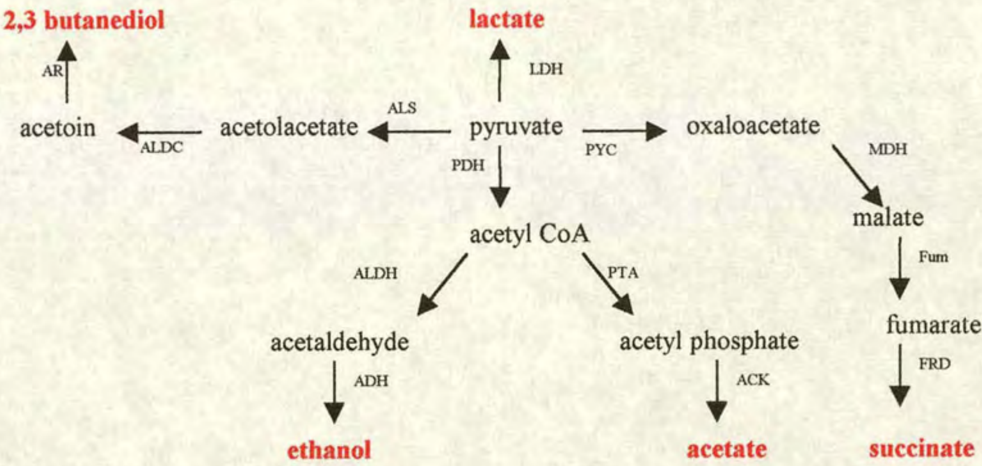


Fig 1.17 Fermentation pathways of *B. subtilis*. The pathways are deduced from identification of the end products (in red) (Nakano *et al.*, 1997). ACK-acetate kinase; ADH-alcohol dehydrogenase; ALDC-acetolactate decarboxylase; ALDH-aldehyde dehydrogenase; ALS-acetolactate synthase; AR-acetoin reductase; FRD-fumarate reductase; Fum-fumarase; LDH-lactate dehydrogenase; MDH-malate dehydrogenase; PTA-phosphotransacetylase; PYC-pyruvate carboxylase; PDH-pyruvate dehydrogenase

In contrast, *E. coli* can ferment either glucose or pyruvate. The proportion of the products excreted depends on the growth conditions, indicating that the cell possesses considerable flexibility in solving its redox problems and the nature of these products varies with species (Lin, 1991). Entry into the fermentative pathway depends on the activity of pyruvate formate-lyase and formate hydrogen-lyase (Alexeeva *et al.*, 2000).

Pyruvate formate-lyase catalyses the transformation of pyruvate to acetyl-coenzymeA and presents an active (Ea) and an inactive (Ei) form. The conversion Ei to Ea is catalysed by an iron(II)-dependent convertor enzyme (PFL-activating enzyme; Conradt *et al.*, 1984). Pyruvate formate-lyase decomposes formic acid to hydrogen and carbon dioxide. It has a molybdenum cofactor, two molybdopterin guanine dinucleotide cofactors, a [4Fe-4S] cluster and a selenocysteine at the active site (Boyington *et al.*, 1997).

Although it has been reported that *Shewanella* spp cannot ferment (MacDonell & Colwell, 1985), Blast searches of the incomplete *Shewanella* MR-1 genome using *E. coli* formate hydrogen-lyase amino acid sequence revealed the presence of similar enzymes. The closest homologues were found in *V. cholerae* (80-83 % identity) and *E. coli* (57-66 % identity). These findings prove that *Shewanella* contains the required DNA sequences to synthesise these enzymes, but whether they are used or not remains to be studied.

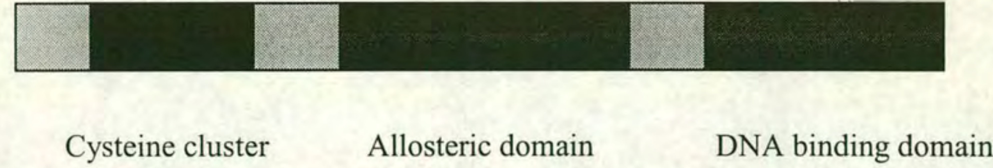
1.5 Respiratory regulator proteins

1.5.1 FNR

FNR (fumarate-nitrate reduction) is a global regulator protein, expressed under both aerobic and anaerobic growth conditions (Spiro & Guest, 1987), which controls gene expression in response to oxygen deprivation. When oxygen is limiting, FNR functions to activate the transcription of genes whose products participate in anaerobic energy generating pathways: nitrate reductase (*narGHJI*), fumarate reductase (*frdABCD*), TMAO/DMSO reductase (*dmsABC*), nitrite reductase (*nirB*), nitrite export (*narK*) and formate dehydrogenase (*fdnGHI*). FNR can also repress the expression of

genes associated with the aerobic electron transport pathways, including cytochrome *o* oxidase (*cyoABCDE*) and cytochrome *d* oxidase (*cydAB*).

There are three domains in the FNR protein (Fig 1.19):



Cysteine cluster Allosteric domain DNA binding domain

Fig 1.19 Schematic representation of the FNR protein

The N-terminal domain, or "the cysteine cluster", contains three cysteine residues. Together with a fourth cysteine residue, located in the allosteric domain, they bind a redox/oxygen sensitive [4Fe-4S] cluster and convert the protein into an active conformation for DNA binding (Green & Guest, 1993a; Green *et al.,* 1993b). Mutational analysis has shown that the four cysteines are required for function (Schwartz *et al.*, 2000). Iron is also required for regulation of FNR activity by oxygen: the transcriptional activating function of FNR can be manipulated by Fe^{2+} levels (Green & Guest, 1993a; Green *et al.,* 1993b) and the anaerobic form of FNR could be converted to an inactive form by varying the redox potential of the growth medium.

The allosteric domain forms a loop-like structure that contacts RNA polymerase (Bates *et al.,* 1995).

The C-terminal domain contains a DNA binding motif that recognises the DNA target site with the consensus sequence TTGAT-N4-ATCAA (the FNR box). This is centred ~41.5 bp upstream from the transcription start point (Darwin *et al.,* 1998).

Anaerobic transcriptional activation by FNR depends on the presence of the redox-sensitive [4Fe-4S] cluster. This cluster is required for oxygen sensing (Unden *et al.,* 1997), and is rapidly destroyed after exposure to oxygen (Bates *et al.,* 2000; Khoroshilova *et al.,* 1996). Absorption and EPR spectroscopies indicated that the native protein contains one [4Fe-4S] cluster that rapidly degrades to two [2Fe-2S] clusters in air (Khoroshilova *et al.,* 1997).

In its active form, FNR may dimerise (Jennings & Beccham, 1993, Melville & Gunsalus, 1996) with a partner molecule and bind strongly to the FNR box. Such organisation of binding sites allows physical contact between the FNR dimer and RNA polymerase. Once this contact occurs, the transcription of a number of target genes essential for anaerobic metabolism can commence.

The *Paracoccus denitrificans* counterpart of the *E. coli fnr* gene is *fnrP*, which contains all functional domains that are found in FNR. A mutation in *fnrP* has pleiotropic effects during growth in conditions of oxygen depletion. The mutant was unable to express membrane-bound nitrate-reductase and cytochrome *c* peroxidase. A second FNR homologue, NNR, controls genes encoding for nitrite- and nitrous oxide reductase (van Spanning *et al.,* 1995). Despite a high degree of identity, NNR and FNR P are not interchangeable.

The ANR homologue from *Pseudomonas aeruginosa* regulates nitrate reduction, HCN production and the arginine deiminase pathway (Gallimand *et al.,* 1991, Zimmerman *et al.,* 1991).

In *Shewanella putrefaciens* MR-1, EtrA is 73.6 % identical to the amino acid sequence of FNR and regulates an essential component of the Fe(III), thiosulfate, sulfite, TMAO, DMSO, nitrite and fumarate reduction pathways (Saffarini & Nealson, 1993).

1.5.2 NarL/NarQ

Respiratory gene expression is also regulated in response to nitrate and nitrite, the preferred anaerobic electron acceptors. Nitrate and nitrite control is mediated by homologous response regulators (NarL and NarP) which communicate with homologous membrane-bound sensor proteins (NarX and NarQ). There are several distinct patterns of operon expression known, including induction by nitrate (for the *narG* operon), repression by nitrate (*frdA* operon), induction by nitrate or nitrite (*nirB* operon) and induction by nitrite and inhibition by nitrate (*nrfA* operon; Darwin *et al.,* 1998). Activation by NarL/Nar P occurs when one or both of these proteins bind upstream of an FNR-binding site centred at -41.5. The *E. coli napF* operon control region is an exception of NarL/NarP dependent regulation: the FNR-binding site is at position -64.5, instead of -41.5 (see 1.5.1), and activation by NarP is mediated by a binding site downstream at position -44.5 (Darwin *et al.,* 1998). Nitrate and nitrite activation is dependent on NarP but is antagonised by the NarL protein (Darwin *et al.,* 1998).

NarX and NarQ control the activity of NarL and NarP via phosphoryl transfer (Schroder, 1994). NarL~P (phosphorylated NarL) fully activates *narG* and the *fdnG* operon expression and fully represses *frdA* and the *nrf* operon. The NarX protein also negatively regulates the NarL protein, by acting as a NarL~P phosphatase in the absence of nitrate (Rabin & Stewart, 1993; Fig 1.20).

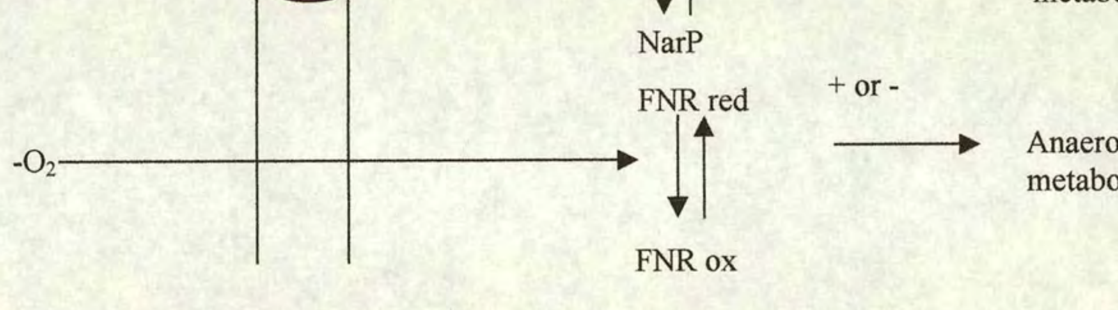


Fig 1.20 Regulatory network involved in oxygen and nitrate regulated gene expression and the response to the signals (Unden & Bongaerts, 1997).

Stimuli: oxygen, nitrate

Sensors: ArcB, NarX, NarQ

Response regulators: ArcA, NarL, NarP and the cytoplasmic DNA sensor-regulated FNR

Activation and repression of the target gene expression is given by + or -

Active phosphorylated states: ArcA~P,NarL~P, NarP~P

1.5.3 ArcB/ArcA

The ArcB/ArcA (aerobic respiration control) system is important in gene expression during anaerobiosis. During anaerobic growth ArcA represses transcription of several genes involved in aerobic metabolism (Iuchi & Kin, 1988). The system also activates transcription of the pyruvate formate-lyase gene (Sawers & Suppman, 1992) as well as the cytochrome *d* oxidase operon (Iuchi *et al.,* 1990a). ArcB is the repressor partner for ArcA (Iuchi *et al.,* 1990b). Upon stimulation, ArcB undergoes two autophosphorylations and transfers a phosphate group to ArcA, which then becomes functional (Iuchi & Lin, 1992). Mutations in either gene derepress several dehydrogenases, the cytochrome *o* complex, members of the citric acid cycle and glyoxylate shunt and the pathway for fatty acid degradation.

1.5.4 ResDE

The *Bacillus subtilis fnr* gene is under the control of a secondary regulatory component, the ResDE system. The *resDE* locus is involved in the regulation of various other central cellular functions, such as sporulation, carbon source utilization and heme *a* synthesis. ResD is required for expression of several components of the aerobic respiratory chain and for anaerobic growth on nitrate (Sun *et al.,* 1996).

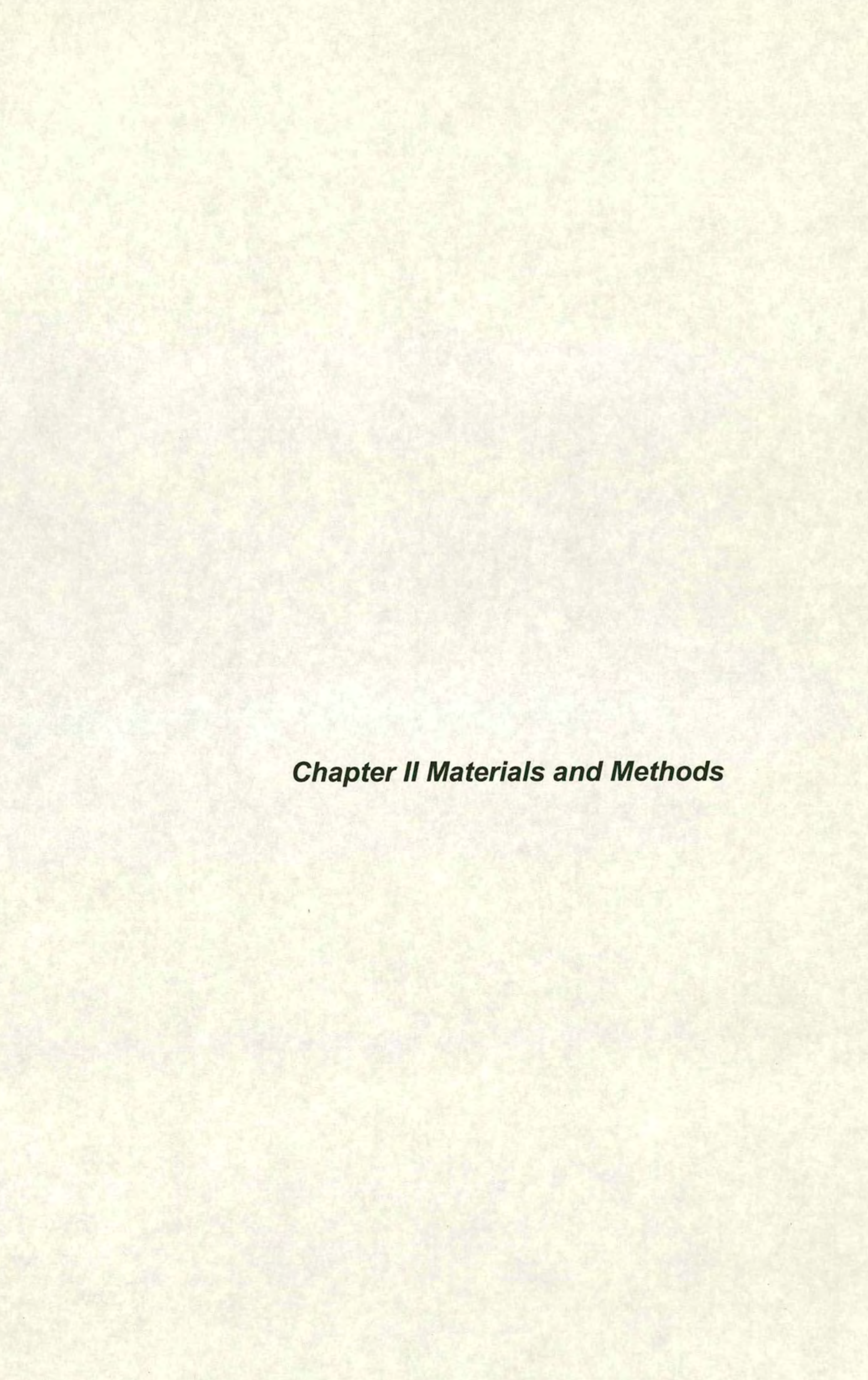
The two-component system encoded by *resDE* is responsible for the induction of respiratory nitrate reductase partially via transcriptional activation of *fnr* (Nakano, 1996). *fnr* mutations totally abolished respiratory growth using nitrate as an electron acceptor (Hoffmann *et al.,* 1998). A dissimilatory nitrite reductase activity which

required *resDE* but not *fnr* for its formation under anaerobic growth conditions was identified and measured (Hoffmann *et al.,* 1998).

Aims

The recent availability of the genomic DNA sequence of *Shewanella* MR-1 would allow us a better insight into the composition of the respiratory chains in this bacterium. The aims of this project were:

1. to identify and classify the *c*-type cytochromes found in *Shewanella* MR-1.
2. Isolation and characterisation of Cyc129. Cyc129 belongs to the outer membrane cytochromes family, a feature found only in *Shewanella* MR-1 and which is thought that it might be involved in iron and manganese oxides reduction. So far, no iron or manganese reductases have been isolated and it is generally believed that one of the outer membrane decaheme cytochromes is the best candidate for this function.
3. Isolation and characterisation of Cyc202, a heptaheme *c*-type cytochrome unique in *Shewanella* MR-1. The presence of seven heme binding sites is not very common in the bacterial world (only *M. capsulatus* contains another heptaheme cytochrome). We presume a novel heme packing in the crystal structure of this protein.
4. Isolation and characterisation of FccA56, a flavoprotein very similar to the flavin domain of fumarate reductase from *S. frigidimarina* NCIMB400. Together with a small *c*-type tetraheme cytochrome, FccA56 resembles the fumarate reductase (currently studied in our lab) and it might complement the EG301 strain that contains a fumarate reductase null mutation.



Chapter II Materials and Methods

2.1 Media

2.1.1 Luria-Broth

Per litre:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

2.1.2 Wood-Baird medium

Per litre:

NaCl	20 g
K_2HPO_4	1 g
Peptone	5 g
Yeast-extract	2 g
$MgSO_4$ $7H_2O$	1 g

2.1.3 Terrific Broth

Per litre:

Bacto-tryptone	12 g
Bacto-yeast extract	24 g
Glycerol	4 ml

2.1.4 Minimal medium

Per litre:

$(NH_4)_2SO_4$	1.2 g
K_2HPO_4	1 g
KH_2PO_4	0.45 g
$NaHCO_3$	0.17 g
$CaCl_2$	0.1 g

The pH was adjusted to 7.4 with NaOH. After autoclaving 50 ml of trace elements and 100 µl of amino acid solution were added.

Trace elements

Per two litres:

H_3BO_3	0.14 g
NaCl	0.0234 g
$CoCl_2$	0.06 g
$NiCl_2$	0.05 g
Na_2MoO_4	0.04 g
SeS_2	0.01 g
$ZnSO_4$	0.02 g
$CuSO_4$	0.002 g
$MnSO_4$	0.02 g

Amino acids (per 100 ml)

Arginine	0.02 g
Glutamate	0.02 g
Serine	0.02 g

Supplements to minimal medium

Electron acceptor	*Final concentration*
Dimethylsulfoxide	10mM
Fe(III)Citrate	50 mM
Fe(III) Chloride	50 mM
Fe(III)Sulfate	50 mM
Fumarate	5 mM
Glycine	5 mM
MnO_2	30 mM
$NaNO_2$	3 mM
$NaNO_3$	15 mM
Na_2SO_3	10 mM
$Na_2S_2O_3$	10 mM
Trimethylamineoxide	25 mM
Transcinnamic acid	5 mM
Trans 3-pyridyl acrylic acid	5 mM
Urocanic acid	5 mM
Indoleacrylic acid	5 mM
Hydroxycinnamic acid	5 mM

Dihydroxybenzoic acid	5 mM
Dimethylfumarate	5 mM
Mesaconic acid	5 mM
Acrylic acid	5 mM
Crotonic acid	5 mM
Tiglic acid	5 mM
Methacrylic acid	5 mM
Senecioic acid	5 mM
Benzoic acid	5 mM
Caffeic acid	5 mM
Ferulic acid	5 mM

Carbon source

Acetate	15 mM
Formate	15 mM
Lactate	15 mM
Succinate	15 mM

When plates were required, agar was added to 3 % (w/v)

2.2 Growth conditions

E. coli strains were grown at 37 ^{o}C and *Shewanella* strains at 23 ^{o}C in LB medium supplemented with the appropriate antibiotics. For anaerobic conditions the "Oxoid" gas-generating system was used.

2.3 Antibiotics

Where required antibiotics were added to the media in the following concentrations: 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 10 μg/ml gentamicin, 50 μg/ml streptomycin, 10 μg/ml rifampicin, 50 μg/ml kanamycin.

2.4 Bacterial strains used

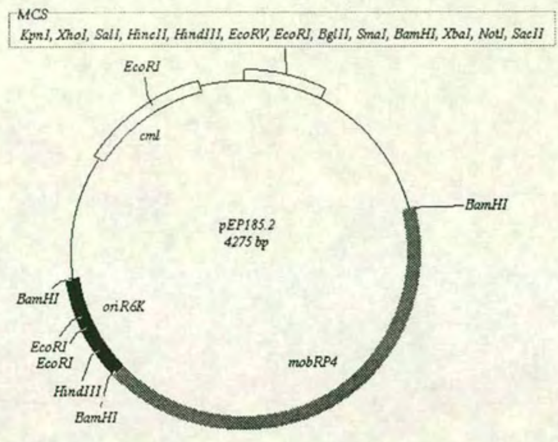
E. coli strains

Strain	***Genotype***	***Source***
DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Lab stock
TG1	supE hsdΔ5 thi (Δlac-proAB) F' [traΔpro AB^+ $lacI^q$ lacZΔM15]	Lab stock
SM10	thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^R	Lab stock
S-17	thi pro hsdR hds M^+recA, RP4(Tc::Mu Km::Tn7)	Lab stock
HMS174	recA1 hsdR rif^R	Lab stock
BL21(DE3)	hsdS gal(λ ci ts857 ind1 Sam7 nin5 lacUV-T7 gene1)	Lab stock
B834(DE3)	F-ompT gal [dcm][lon] $hsdS_B(r_B^-, m_B^-)$ met (DE3)	Lab stock

Shewanella strains

Strain	*Genotype*	*Source*
MR-1	wild type	Lab stock
MR-1 Rf^r	spontaneous rifampicin resistant strain	Lab stock
DA129	*Shewanella* MR-1 Rf^r harbouring pex129	This study
DA202	*Shewanella* MR-1 Rf^r harbouring pex202	This study
DAfccA56	*Shewanella* MR-1 Rf^r harbouring pexfccA56	This study
Δ129	MR-1 Rf^r Δ129::ahp Km^r	This study
Δ202	MR-1 Rf^r Δ202::ahp Km^r	This study
ΔfccA56	MR-1 Rf^r ΔfccA56::ahp Km^r	This study

2.5 Plasmids used



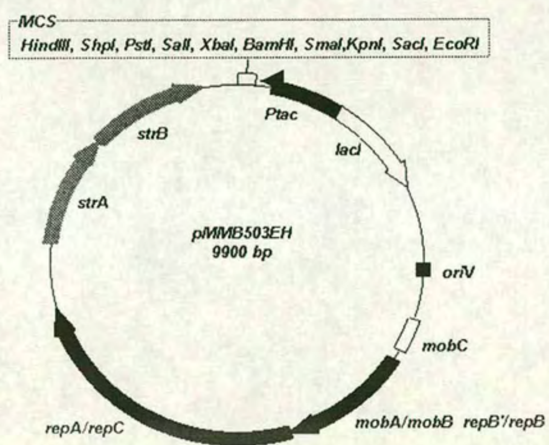


Fig 2.1 Selected plasmid maps.

pEP185.2 is a suicide vector used for gene inactivation (Pepe & Miller, 1993)

pMMB503EH is a broad host range expression vector used for protein overexpression in *Shewanella* MR-1

Plasmid	**Description**	**Source/Reference**
pGEM-T	3003 bp cloning vector Ap^r	Promega
pT7-7	2437 bp expression vector, T7RNA polymerase ϕ10 promoter	Lab stock
pMMB503EH	9.9 kbp broad host range expression vector, *tac* promoter Sm^r	Michel *et.al* (1995)
pJQ200KS	5368 bp suicide vector, *ori* p15A, *mob*RP4, Gm^r	J. Quandt *et.al* (1993)
pEP185.2	Suicide vector, *ori*R6K, *mob*RP4, Cml^r	Pepe & Miller, 1993
pEC86	Helper plasmid containing the *ccm* genes	Arslan *et al*., 1998
pBluescriptSK+	2956 bp cloning vector, Ap^r	Lab stock
pRS551	12460 bp *lac*Z, *lac*Y, Ap^r, Km^r	R.W. Simons (1987)
pSUP10121	Broad host range mobilization vector Km^r	Berg & Berg (1983)
pDA2	2300 bp *cyc129* gene in pGEM-T	This study
pDA3	2100 bp *cyc202* gene in pGEM-T	This study
pDA4	1500 bp *fccA56* gene in pGEM-T	This study
pDA5	347 bp *fccB56* gene in pGEM-T	This study
pDA6	1839 bp *fccA56+fccB56* genes in pGEM-T	This study
pDA7	1950 bp *hal56* gene in pGEM-T	This study
pDA8	3823 *bp fccA56+fccB56+hal56* in pGEM-T	This study
pDA6-2	1839 bp *Bam*HI fragment from pDA6 in pT7-7	This study
pDA2-2	pDA2 cut with *Psh*AI/*Msc*I and recircularized	This study
pDA3-2	pDA3 cut with *Bgl*II/*Eco*RI and recircularized	This study
pDA4-2	pDA4 cut with *Bsu*36I/*Xba*I recircularized	This study
pDA5-2	pDA4 cut with *Bsl*I/*Bsr*GI and recircularized	This study
pDA2-3	*traJ+sacB*+Gm^r from pJQ200KS cloned into pDA2-2	This study

pDA3-3	*traJ*+*sacB*+Gm^r from pJQ200KS cloned into pDA3-2	This study
pDA4-3	*traJ*+*sacB*+Gm^r from pJQ200KS cloned into pDA4-2	This study
pDA2-4	1338 bp *Bam*HI/*Sal*I fragment from pDA2-2 in pJQ200KS	This study
pDA3-4	1188 bp *Bam*HI/*Sma*I fragment from pDA3-2 in pJQ200KS	This study
pDA4-4	558 bp *Bam*HI/*Sma*I fragment from pDA4-2 in pJQ200KS	This study
pDA3-5	2100 bp *202* gene in pSK+	This study
pex129	2300 bp *129* gene in pMMB503EH	This study
pex202	2100 bp *202* gene in pMMB503EH	This study
pexfccA56	1500 bp *fccA56* gene in pMMB503EH	This study
pDA2-6	pDA2-2 *129*::*ahp*	This study
pDA3-6	pDA3-2 *202*::*ahp*	This study
pDA4-6	pDA4-2 *fccA56*::*ahp*	This study
pDA2-7	2735 bp *Apa*I/*Pst*I fragment from pDA2-6 in pEP185.2	This study
pDA3-7	2627 bp *Apa*I/*Pst*I fragment from pDA3-6 in pEP185.2	This study
pDA4-7	2352 bp *Apa*I/*Pst*I fragment from pDA4-6 in pEP185.2	This study
pDA4-8	1500 bp *Bam*HI/*Pst*I fragment from pDA4 in pT7-7	This study
pDA4-9	24 bp *Nde*I/*Bam*HI fragment eliminated from pDA4-8	This study
pDA129-Km	1.5 kb fragment from inverse PCR with Δ129(?) template	This study

pDA56-Km	1.5 kb fragment from inverse PCR with ΔfccA56(?) template	This study
pCM51	900 bp *Eco*RV Km^r fragment from pSUP10121 in pGEM-T	This study
pCM2-10	pDA2 *129*::*ahp*	This study
pCM3-10	pDA3 *202*::*ahp*	This study
pDA4-10	pDA4 *fccA56*::*ahp*	This study
pDA2-11	*129*::*ahp Sac*II/*Sal*I fragment from pDA2-10 in pEP185.2	This study
pDA3-11	202::ahp *Sac*II/*Sal*I fragment from pDA3-10 in pEP185.2	This study
pDA4-11	*fccA56*::ahp *Sac*II/*Sal*I fragment from pDA4-10 in pEP185.2	This study
pDA4-81	*fccA56*-His tag in pGEM-T	This study
pDA4-84	*fccA56* without signal sequence and His tag in pGEM-T	This study
pDA4-85	*fccA56* without signal sequence and His tag in pT7-7	This study

2.6 Primers used

Name	Sequence 5'-3'
129L	CACGGATCCATGATGAAAAACTAC
129R	CACGAATTCCTAGTGTGCTACCTCTGC
202L	CACGGATGGATGATGAAGCGATGG
202R	CACCCCGGGCTACATTTTAGCGTT
A56L	CACGGATCCATGGCCGGAAGAAGA
A56R	CACCCCGGGCTACACTTTTCTAATGGC
A56-His	TCTTAGTGGTGGTGGTGGTGGTGAGCCACTTTTCTAATGGC
A56-noSS	CACCATATGATGGCTATGGCATTT
fccB56L	CACCCCGGGATGATGAAATATA
fccB56R	CACGGATCCCTTTAGATTTGGA
fccB54L	CACCCCGGGATGATGAAAACATTAC
fccB54R	CACGGATCCTACCATTTCTTTGAACCTT
REVKAN1	CACTTTCCCGTTGAATATGGCTC
REVKAN2	CACGCACTTTCATTTGATGCTCGATGAG
Tn5Km(L)	CACGATATCCCGGAATTGCCA
Tn5Km(R)	CACGATATCTCAGAAGAACTC
HAL56(L)	CCCGAATTCATGAAATTAAAAACTATACCGCTTG
HAL56(R)	CACGCATGCTTATCTTGTACTGTAGGTTTTT

2.7 Bacterial methodology

2.7.1 Preparation of competent cells

LB medium (5ml) was inoculated with the appropriate *E. coli* strain and incubated at 37 ºC until OD_{600}~0.5. The cells were chilled on ice (10 min) and centrifuged (10 min, 5000 rpm). The pellet was resuspended in ice cold $CaCl_2$ (5 ml,

100 mM) and chilled on ice for 30 minutes. The cells were pelleted and resuspended in 1 ml of 100 mM $CaCl_2$.

2.7.2 Transformation

The ligation mixture was diluted with 10 μl TE buffer and 200 μl of competent cells added. After incubation on ice for 30 minutes, the cells were heat-shocked for 80 sec at 42 ºC, before being placed on ice for 5 minutes. LB medium (800 μl) was added and the tubes incubated at 37 ºC for 1 hour with shaking. The transformed cells were spread on agar plates supplemented with the appropriate antibiotics.

2.7.3 Bacterial conjugation

The filter method of cell mating was used. Bacterial cells were grown on LB medium with the appropriate antibiotics to mid-log phase (OD_{600}~0.8). Donor and receptor cells were mixed in different ratios (1:10 usually) and spotted onto sterile 0.2 μm nitrocellulose filters (Whatman) on LB agar plates and incubated overnight at 23 °C. The filters were then vortexed in 1 ml LB medium and the cells pelleted. The pellet was resuspended in LB medium and plated on agar plates supplemented with the appropriate antibiotics.

2.7.4 Preparation of periplasm

E. coli or *Shewanella* strains were grown in Wood-Baird medium to an OD_{600nm}~0.5 (0.8 for *Shewanella*). The cells were induced with IPTG and grown for another 3 hours. The cells were harvested by centrifugation for 15 minutes at 9000

rpm and washed in buffer A. The pellet was resuspended in buffer B and incubated statically at room temperature for 10 minutes. Lysozyme was added to a final concentration of 30 μg/ml and the cells incubated for a further 10 minutes. An equal volume of buffer C was added and the mixture stirred continuously to shock the cells. After 10 minutes EDTA was added to a final concentration of 1 mM. The formation of the spheroplasts was followed by phase contrast microscopy. The reaction was stopped with $MgSO_4$ at a final concentration of 1 mM. The shocked cells were centrifuged at 30000 rpm for 30 minutes and the periplasm separated from the pellet.

Buffer A	**Buffer B**	**Buffer C**
10 mM Tris-HCl pH 7.5	100 mM Tris-HCl pH 8.0	10 mM Tris-HCl
0.1 M NaCl	0.5 M sucrose	pH 8.0

2.7.5 Blue / white colonies screening

The screening of recombinant from non-recombinant vectors in cloning experiments was facilitated using vectors that carry the *lacZ* gene (pSK+, pGEM-T). Recombinant vectors form blue colonies, due to X-Gal metabolised in the presence of IPTG. 40 μl of a 40 mg/ml solution of X-Gal in dimethylformamide and 4 μl 1M IPTG solution was spread on the surface of the plates, prior to plating out the cells.

2.8 DNA techniques

2.8.1 Genomic DNA extraction

A 2 ml aliquot of an overnight culture was centrifuged and the pellet resuspended in 10 mM EDTA pH 8. The cells were lysed by adding SDS to a final concentration of 0.2 %. The lysate was extracted with phenol, followed by a re-extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with 2 volumes of ethanol and 0.1 volumes of 3 M sodium acetate pH 5.2 and resuspended in distilled water.

2.8.2 Plasmid DNA preparation

An overnight culture (5 ml) was centrifuged and the pellet resuspended in TEG buffer; the cells were lysed by the addition of alkaline lysis buffer and incubation on ice for 5 minutes. The tubes were centrifuged and the supernatant extracted with phenol:choloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with 2 volumes of ethanol (100 %) and 0.1 volumes of 3 M sodium acetate pH 5.2 and resuspended in TE buffer. For low copy number plasmids, a 10 ml overnight culture was used.

TEG buffer	**TE buffer**	**Alkaline lysis buffer**
25 mM TRIS pH 8	10 mM TRIS pH 8	0.2 M NaOH
10 mM EDTA pH 8	0.1 mM EDTA pH 8	0.2 % SDS
50 mM glucose		

2.8.3 Single stranded DNA preparation

A mid-log phase culture (2 ml) containing a phagemid (plasmids that carry the origin of replication of the filamentous bacteriophage f1) was infected with 1.4 μl M13KO7 helper phage (Promega, at a multiplicity of infection of 10) and incubated for 80 minutes at 37 °C. A 400 μl aliquot of this culture was transferred to a flask containing 10 ml LB supplemented with 70 μg/ml kanamycin and incubated with good aeration overnight. The culture (1.5 ml) was centrifuged and the phagemid DNA precipitated with 300 μl of 2 M NaCl / 20 % PEG8000 for 15 minutes at room temperature. The pellet was resuspended in 200 μl TE buffer and extracted with phenol and chloroform. The aqueous phase was transferred to a fresh tube and the DNA precipitated with 0.5 volumes 3 M sodium acetate pH 5.2 and 2 volumes of ethanol (100 %) for 1 hour at –20 °C. After centrifugation (10 min at 30000 rpm), the DNA was resuspended in 20 μl dH_2O.

2.9 DNA fragment preparation (Qiagen gel extraction kit)

The DNA fragments resulting from single or double digests were run on a 0.8 % high purity agarose gel (Appligene) and the fragment of interest was excised. The gel slice was incubated at 50 °C with 3 volumes of QXI buffer and 10 μl of QiaexII resin. Following the solubilisation of the agarose, the mixture was centrifuged (1 min at 30000 rpm) and the pellet washed again with QXI buffer and twice with buffer PE. The DNA was eluted in 20 μl dH_2O.

2.10 Ligation of DNA fragments

After the vector and insert had been cut with the appropriate restriction enzymes and the fragments purified as described before, the concentrations were estimated by agarose gel electrophoresis against a DNA of a known concentration (1 kb DNA marker, GibcoBRL). Ligations were performed in 10 μl (1x) reaction buffer (Promega) using 1U T4 DNA ligase (Promega), 50 ng vector and 150 ng insert. The mixture was incubated overnight at 16 °C.

2.11 Converting 5' and 3' over-hangs to blunt ends

The DNA fragment of interest was purified as described before and eluted in 1x T4 DNA polymerase buffer supplemented with 100 μM of each dNTP and 0.1 mg/ml BSA. T4 DNA polymerase (5U) was added and the mixture incubated for 20 min at 16 °C. The reaction was stopped by heating for 10 minutes at 75 °C.

2.12 DNA amplification

2.12.1 Polymerase chain reaction

DNA amplification was performed using 5 pmol of each primer, 0.5 μg genomic DNA and 1.25 U Taq polymerase (Promega, Böehringer-Mannheim, or BioLine) in a 50 μl reaction mix. The mixture was covered with 20 μl mineral oil to prevent evaporation.

Reaction mix: 10 mM TRIS-HCl pH 8.3

50 mM KCl

3 M $MgCl_2$

200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)

2.12.2 Colony PCR

One bacterial colony was resuspended in 50 μl dH_2O and boiled for 5 minutes. 5 μl of the lysate was used to perform a PCR reaction under the conditions described above.

2.12.3 Inverse PCR

Genomic DNA was digested with different restriction enzymes. After the enzymes were inactivated, the fragments were religated overnight at 16 °C with 1 U T4 DNA ligase (Promega). The ligase was inactivated by heating for 10 minutes at 70 °C. 5 μl of the ligation mixture was used for a PCR reaction under the conditions described before.

2.13 DNA sequencing

2.13.1 Manual sequencing

Single stranded DNA was annealed to 1 pmol oligonucleotide primer by heating to 65°C for 2 minutes and slowly cooling down to room temperature. To the annealing mixture 2 μl Sequenase Version 2.0 T7 DNA polymerase (Amersham) was added. The mixture was incubated for 5 minutes at room temperature and 3.5 μl were added to 4 tubes, each containing 2-5 μl pre-warmed termination reaction (ddNTP). After 5 minutes of incubation at 37 °C, the reaction was stopped by the addition of 4 μl stop solution.

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Labelling mix (dGTP): 7.5 μM dGTP

7.5 μM dCTP

7.5 μM dTTP

Termination nucleotide mix (one for each dideoxynucleotide).

Each contains 80 μM dATP

80 μM dCTP

80 μM dGTP

80 μM dTTP

In addition the A mix contains 8 μM ddATP, the C mix contains 8 μM ddCTP, the G mix 8 μM ddGTP and the T mix 8 μM ddTTP.

Stop solution: 95 % formamide

20 mM EDTA

0.05 % bromophenol blue

0.05 % xylene cyanol

Enzyme dilution buffer: 10 mM TRIS-HCl pH 7.5

5 mM DTT

0.5 mg/ml BSA

2.13.2 Denaturing gel electrophoresis

Urea (25.2 g) was disolved in 12 ml of 30 % acrylamide, 6.5 ml 10xTBE and the volume adjusted to 65 ml with dH_2O. After 30 minutes of continuous stirring, 500 μl of 10 % ammonium persulfate and 100 μl TEMED were added. The gel was kept on a flat surface overnight and pre-run for 1 hour. For a 200 bp reading the gel was run for 2 hours at 65 W.

After running, the gel was soaked for 5 minutes in 5 % acetic acid (v/v) and 15 % methanol (v/v) to remove the urea. After drying the gel was exposed to an X-ray film (AGFA) for 1-2 days before developing.

2.13.3 Automated sequencing

0.1 μg/μl single-stranded DNA or 0.2 μg/μl double stranded DNA were mixed with 8 μl Terminator Ready Mix (Perkin Elmer Applied Biosystem), 3.2 pmol primer and water to 20 μl. The reaction mix was covered with mineral oil and subjected to a thermal cycling, for 25 cycles (10 sec at 96 °C, 5 sec at 50 °C, 4 min at 60 °C). The DNA was precipitated with 0.5 volumes 3 M sodium acetate and 2 volumes of ethanol (100 %) overnight at –20 °C and the pellet rinsed with 75 % ethanol. Samples were processed by the ICMB Automated Sequencing service on an ABI377 instrument.

Terminator Premix: A-Dye terminator, C-Dye terminator, G-Dye terminator, T-Dye Terminator

dITP, dATP, dCTP, dTTP

TRIS-HCl pH 9

$MgCl_2$

Thermal stable pyrophosphatase

AmpliTaq DNA polymerase

2.14 Detection of homologous DNA fragments

2.14.1 Southern blot

Genomic DNA was cut with one or more restriction endonucleases according to the manufacturers instructions (Promega, New England BioLabs). Samples were separated electrophoretically on a 0.8 % agarose gel. The DNA was denatured for 45 minutes in denaturing solution and then neutralised for 30 minutes in neutralisation solution. The DNA was transferred from the gel to a nylon membrane by capillary transfer overnight. The DNA attached to the filters was UV cross-linked and pre-hybridised.

2.14.2 Pre-hybridisation and hybridisation with radioactive probe (^{32}P)

The pre-hybridisation was carried out at 65 °C in the pre-hybridisation solution. After 2 hours, the radio-labelled probe was added and the filters incubated for 12-14 hours at 65 °C.

2.14.3 Detection

After hybridisation, the filters were washed in 2x washing solution for 10 minutes at room temperature and 0.1x washing solution for 15 minutes at 65 °C. The filters were exposed to X-ray film for 4-24 hours.

Denaturing solution	**Neutralising solution**	**20X SSC**
1.5 M NaCl	1M TRIS-HCl pH 7.4	3 M NaCl
0.5 M NaOH	1.5 M NaCl	0.3 M sodium citrate

Pre-hybridisation solution	**Denhardt solution**
6x SSC	2 % BSA
5x Denhardt sol	2 % ficoll
0.5 % SDS	2 % polyvinyl pyrrolidone
100 μg/ml sonicated non-homologous DNA	

2 x washing solution	**0.1x washing solution**
2 x SSC	0.1x SSC
0.1 % SDS	0.1 % SDS

2.14.4 DNA radio-labelled probe

Double stranded DNA samples used as probes for Southern hybridisation were radio-labelled using a "Nick Translation System" kit (Promega). Nicks were made into the DNA template by DNaseI, producing exposed 3' hydroxyl termini. The radio-labelled nucleotide was incorporated at the position where the original

nucleotide was excised. 1.5 μg DNA were radio-labelled in 50 μl of labelling mixture. The mixture was incubated at 15 °C for 1 hour and stopped with 25 mM EDTA pH 8. The probe was heat-denatured for 10 minutes before being added to the hybridisation solution.

Labelling mixture: 200 Ci / mmol of ^{32}P

500 mM TRIS-HCl pH 7.2

100 mM $MgSO_4$, 1 m M DTT

100 μM dNTP

5 U DNA polymerase I

2.14.5 Pre-hybridisation and hybridisation with DIG labelled probe (Amersham)

The blot was placed in a hybridisation bag containing 20 ml standard buffer/100 cm^2 membrane and pre-hybridised for 2 hours at 65 °C. The pre-hybridisation solution was replaced with the hybridisation solution and the blot was incubated at 65 °C for 14-18 hours. Upon completion of hybridisation the probe was removed and the blot washed for 2x 5 minutes with 2x wash solution at room temperature and 2x15 minutes with 0.5x wash solution at 65 °C.

Standard buffer	**2x wash solution**	**0.5x wash solution**
0.1 % (w/v) N-lauroylsarcosine	2 x SSC	0.5 x SSC
1 % blocking reagent	0.1 % SDS	0.1 % SDS

2.14.6 Chemiluminescent detection

After the post-hybridisation washes the membrane was equilibrated in washing buffer for 1minute. The membrane was blocked in blocking solution; after 30 minutes the solution was replaced by Anti-Digoxigenin-AP solution and incubated for 30 minutes. The antibody solution was discarded and the membrane washed for 2x 15 minutes in washing buffer. CSPD substrate was added and incubated for 5 minutes at room temperature. The membrane was exposed to an X-ray film for 5-20 minutes at 37 °C.

Washing buffer	**Maleic acid buffer**	**Blocking solution**
100 m M maleic acid	100 mM maleic acid	1 %(w/v) stock solution
150 mM NaCl	150 mM NaCl	diluted in maleic acid buffer
pH 7.5	pH 7.5	0.3 % (v/v) Tween

Detection buffer	**Antibody solution**
100 mM TRIS-HCl	1:10000 anti-DIG alkaline phosphatase
100 mM NaCl	diluted in 1 % blocking solution
pH 9.5	

2.14.7 DIG-labelled probe (Random Primed DNA with DIG High Prime)

1 µg DNA template was diluted to 16 µl with dH_2O, heat denatured for 10 minutes and quickly chilled on ice. 4 µl of DIG-High Prime Mix were added and the mixture incubated for up to 20 hours at 37 °C. The reaction was stopped with 20 mM EDTA pH 8.

2.15 SDS polyacrylamide gel electrophoresis of proteins

A mini-Protean Gel electrophoresis kit (BioRad) was used to separate proteins according to their molecular weight. Two layers of polyacrylamide gels were used: a 10 % resolving gel and a 5 % stacking gel. The resolving gel (5 ml) was poured between two vertical glass plates which were clamped together. After polymerisation was complete the stacking gel was poured and the comb inserted. Whilst the stacking gel was polymerising, the samples were boiled for 5 minutes and run in 1x glycine electrophoresis buffer at 8 V/cm gel, alongside a pre-stained broad-range protein marker (NEB). The gel was stained with Coomassie Brilliant Blue for 1 hour and destained in destaining solution.

Resolving gel	**Stacking gel**
Per 5 ml:	Per 1ml
1.9 ml dH_2O	0.68 ml dH_2O
1.7 ml 30 % acrylamide mix	0.17 ml 30 % acrylamide mix
1.3 ml 1.5 M TRIS (pH 8.8)	0.13 ml 1M TRIS (pH 6.8)
0.05 ml 10 % SDS	0.01 ml 10 % SDS
0.05 ml 10 % ammonium persulfate	0.01 ml 10 % ammonium persulfate
0.002 ml TEMED	0.001 ml TEMED

1x SDS gel-loading buffer	**5x TRIS glycine electrophoresis buffer**
50 mM TRIS-HCl pH 6.8	25 mM TRIS pH 8.3
100 mM DTT	250 mM glycine
2 % SDS	0.1 % SDS
0.1 % bromophenol blue	
10 % glycerol	

Coomassie Brilliant Blue stain	**Destaining solution**
0.25 % (w/v) Coomassie Brilliant Blue R250	45 % methanol
in destaining solution	45 % dH_2O
	10 % glacial acetic acid

2.16 Heme staining

After running the gel was equilibrated for 5 minutes in solution A and then transferred to TMBZ solution. After 15 minutes incubation in the dark, hydrogen peroxide was added (1 ml, 30 %); when sufficient colour developed, the reaction was stopped by rinsing with dH_2O.

Solution A	**TMBZ solution**
30 % methanol	400 µg/ml TMBZ in solution A
0.25 M sodium acetate pH 5.2	

2.17 Western blot analysis

The proteins were separated on an SDS-PAGE gel and transferred to a nitrocellulose membrane by electroblotting for 2 hours at 0.6-2 Amps in 1x transfer

buffer. The membrane was placed in milk powder (100 ml, 20 %) made up in TBS and incubated overnight. The blocked membrane was placed in 5 % milk solution (20-40 ml), with 30 μl antiserum and incubated with shaking for 3 hours. The membrane was rinsed for 4x5 min with TBS and then incubated with 20 ml milk solution containing 10 μl HRP (horse radish peroxidase) conjugated antibody for 2 hours. The enzyme (HRP) conjugated to the secondary antibody catalyzes a colorimetric reaction in the presence of the developing solution, resulting in the deposition of coloured substrate on the membrane at the reaction site.

TBS	**10X transfer buffer**	**Developing solution**
10 mM TRIS-HCl pH 7.5	250 mM TRIS-HCl pH 8.3	0.5 mg/ml dianisidine
100 mM NaCl	1.5 M glycine	1 ml 0.1M imidazole pH 7.4
		0.1 ml 30 % H_2O_2
		8.4 ml dH_2O

2.18 Raising antibodies

After purification, 600 μg of FccA56 protein were loaded on a 12 % SDS-PAGE gel and run until enough separation between the protein of interest and other contaminants was obtained. The gel was stained (1 hour) with aqueous Coomassie Blue (0.25 % w/v Coomassie Brilliant Blue R250 in dH_2O), rinsed (3x 10 min) with dH_2O and destained with 1x running buffer (without SDS). The band was cut and placed in a pre-chilled mortar. Using liquid nitrogen, the band was ground to a fine powder that would easily pass through a needle. Using a piece of cold paper, the powder was transferred to a 1.5 ml Eppendorf. When all of the antigen was in the

tube, an equal volume of 1x PBS buffer was added. The sample was aliquoted into 4-5 tubes (the size of the aliquots should not exceed 250 μl).

The immunization was carried out by Dr Maggie Chambers at "Diagnostic Scotland".

1x PBS

Per 1 litre: 8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

pH 7.4

2.19 Flavoprotein FccA56 purification

2.19.1 Culture growth

An overnight culture containing pDA4-9 or pDA4-85 was used to inoculate 5x 2 litre flasks containing 1litre of TB+ampicillin medium. The culture was grown with good aeration at 37 °C until $OD_{600\ nm}$~ 0.5. The protein production was induced overnight (100 μM IPTG/ 30 °C/ 70 rpm).

2.19.2 Preparing bacterial lysate for protein purification

After induction the cells were harvested by centrifugation at 8000 rpm for 10 minutes and 1 g of pellet was resuspended in 5 ml 10 mM phosphate buffer pH 7. Where required, the protease inhibitors PMSF and benzamidine were added (10 μM). The cells were sonicated for 1 min per gram of pellet and centrifuged for 2 hours at 13000 rpm. The supernatant was transferred to a fresh tube and the DNA precipitated

with 0.4 % PolyminP before being centrifuged for a further hour. At this stage the cell lysate was ready for purification by column chromatography.

200 mM Na_2HPO_4

Per 100 ml : 3.56 g $Na_2HPO4 \cdot 2H_2O$

200 mM NaH_2PO_4

Per 100ml: 3.12 g $NaH_2PO_4 \cdot 2H_2O$

100 mM phosphate buffer pH 7

Per 100 ml: 39 ml NaH_2PO_4 (200 mM)

61 ml Na_2HPO_4 (200 mM).

2.19.3 Anionic exchange chromatography

Anionic exchangers exploit the different net charges on proteins at a given pH. At pH 7, FccA56 is predicted to be positively charged and it should not bind to the positively charged matrix.

The cell extract was loaded onto a 20 ml DEAE-Sephacel column (Pharmacia) equilibrated with 10 mM phosphate buffer pH 7. The column was washed with 3 column volumes of buffer. The collected effluent was loaded onto a hydroxyapatite column (BioRad Chemicals).

2.19.4 Hydroxyapatite column

Hydroxyapatite is a calcium hydroxide-phosphate absorbent that has a crystalline surface made up of charged ions, associated with water of hydration. Electrostatic interactions can therefore occur between the protein and the inorganic gel.

A 15-20 ml column (BioRad Chemicals) was equilibrated in 10 mM phosphate buffer pH 7. The effluent collected from the DEAE-Sephacel column was loaded onto the hydroxyapatite and a distinct yellow ring formed at the top of the column. The protein was eluted in steps with 0-5 % $(NH_4)_2SO_4$ in 10 mM phosphate buffer pH 7.

2.19.5 Nickel column (Pharmacia)

His-tagged proteins contain a stretch of 6-10 consecutive histidine residues, that can be expressed at the N- or C-terminal ends of the target protein. The His-tag sequence binds to divalent cations (Ni^{2+}) immobilized on a His-Bind metal chelation resin.

For the His-tagged FccA56 protein, the pellet from 2 litres of culture was resuspended in 20 ml ice-cold 1x Binding Buffer and sonicated as described before. After centrifugation (1 hour at 14000 rpm) the protein lysate was filtered using a 0.45 µm sterile filter (Whatman) and loaded onto the Ni^{2+} affinity column equilibrated in 1x Binding Buffer. The column was washed with 10 volumes of 1x Binding Buffer and 6 volumes of 1x Wash Buffer. The protein was eluted with 6 volumes of 1x Elution Buffer.

8x Binding Buffer	**4x Elution Buffer**	**8x Washing Buffer**
40 mM imidazole	4 M imidazole	480 mM imidazole
4 M NaCl	2 M NaCl	4 M NaCl
160 mM TRIS-HCl	80 mM TRIS-HCl	160 mM TRIS-HCl
pH 7.9	pH 7.9	pH 7.9

2.20 Kinetic analysis

2.20.1 Reduction assay

The ability of FccA56 to reduce different substrates under anaerobic conditions was monitored using a Shimadzu UV-PC-1201 spectrophotometer contained in a glove box maintained at 25 °C and less than 6 ppm O_2. The substrate dependent reoxidation of reduced methyl viologen was monitored at 600 nm in a 0.5 M TRIS-HCl buffer, pH 7.2. The viologen was reduced by addition of sodium dithionite until an absorbance reading of ~1 was obtained. Substrate was added and the reaction initiated by the addition of the enzyme to a final concentration of 5 μM. All substrates were ajusted to pH 7 with 4 M NaOH.

The substrates used are as follows: tiglic acid, crotonic acid, mesaconic acid, methacrylic acid, acrylic acid, 3 β indoleacrylic acid, secioic acid, benzoic acid, *trans*-cinnamic aicd, p-coumaric acid, caffeic acid, ferulic aicd, *trans*-pyridyl acrylic acid, urocanic acid, dihydroxybenzoic acid, fumarate and dimethylfumarate.

2.20.2 Oxidation assay

The dehydrogenase activity was measured by DCIP reduction followed by the decrease in absorbance at 600 nm.

The electron acceptor used (dichloroindophenol, from Sigma) was prepared as a 4 mM stock with phenazine methosulfate (0.27 mM) added. The stock (40 μM) and substrate (0-10 mM) were added to the assay buffer (see 2.20.1). The reaction was initiated by the addition of the enzyme (5 μM) and monitored at 600 nm (Shimadzu UV-PC-1201 spectrophotometer) over a period of 5 minutes. As before, the substrates were prepared as pH 7 solutions.

Substrates used: succinic acid, malic acid, methylsuccinic acid, aspartic acid, asparagine, alanine, serine, histidine, arginine, leucine, glutamic acid and glutamine.

2.21 Potentiometric titration

Potentiometric titrations were conducted in a glove box. The enzyme solution (2 ml of 40 μM active enzyme) was loaded onto a Sephadex G-25 gel filtration column equilibrated in 10 mM Tris + 150 mM NaCl + 10 % glycerol. Soluble mediators were added (~10 μM each) and the solution was titrated using sodium dithionite (0.5 μl, 25 mM) as reductant. After addition of each aliquot 10-15 minutes equilibration time was allowed. Spectra were recorded on a Shimadzu 1201 UV-vis spectrophotometer over the range 600-300 nm. The electrochemical potential of the sample solution was monitored using a CD740 meter coupled to a Pt/calomel electrode at 25 °C.

The following mediators were added to a final concentration of 10 μM:

Mediator	**Mid point potential (mV)**
2-hydroxy-2,4-naphtoquinone	-140
Antraquinone-2-sulfonate	-225
Benzyl viologen	-359
Flavin mononucleotide	-207
Methyl viologen	-430
Phenazine methosulfate	+80

Chapter III Genome Analysis

3.1 Cytochromes *c*

The *c*-type cytochromes are a large and diverse group of proteins that serve as components of the electron transport chains in bacteria, chloroplasts and mitochondria and have one or several heme *c* groups (see Chapter 1.2.3), covalently attached via two thioether bonds, which are formed from the two vinyl groups of the heme and two cysteine residues provided by the sequence motif Cys-x-x-Cys-His, where x is any amino acid and the histidine provides the fifth heme ligand.

Ambler (1991) recognized four classes of *c*-type cytochromes:

Class I includes the low-spin, soluble, monoheme cytochrome *c* of mitochondria and bacteria. The heme attachment site is situated towards the N-terminus and the sixth ligand is provided by a methionine residue, about 40 residues further on, towards the C-terminus. This class includes cytochrome c_{554} from *Paracoccus denitrificans* and cytochrome c_5.

Class II includes the high-spin cytochrome *c*' and various low-spin cytochromes. The heme attachment site is close to the C-terminus. In the low-spin proteins, the sixth ligand is a methionine residue close to the N-terminus.

Class III includes the low-potential, multiple heme cytochromes: cytochrome c_7 (triheme), c_3 (tetraheme) and high-molecular-weight cytochrome *c* (hexadecaheme). The heme *c* groups present different redox potentials in the range of 0 to -400 mV.

Class IV was originally created to hold the complex proteins that have other prosthetic groups as well as heme *c*, like flavocytochrome *c* and cytochrome cd_1.

3.2 Genome analysis

As discussed earlier (see Chapter 1.1) *Shewanella* is a Gram negative, facultative anaerobe isolated from a diverse range of habitats and which displays an incredible versatility. It can couple its anaerobic growth and link respiratory proton translocation to the reduction of a variety of compounds including manganese (IV) and iron (III) oxides, fumarate, nitrate, nitrite, thiosulfate, sulfur, TMAO and DMSO. This versatility implies the existence of a complex electron transport chain. Indeed, analysis of the *S. putrefaciens* MR-1 genome sequence (available at www.tigr.org) reveals the presence of a large number of *c*-type cytochromes and other respiratory enzymes.

To identify these proteins, a series of steps was followed:

1. Blast search of the MR-1 genome with a series of sequences of known *c*-type cytochromes.

2. *Shewanella* MR-1 DNA sequence retrieval from the database (//www.tigr.org).

3. Search for open reading frames (//www.ncbi.nlm.nih.gov/gorf).

4. Blast search (//www.ncbi.nlm.nih.gov/Blast) of each frame against general databases to identify proteins similar to known respiratory enzymes.

5. Analysis of these proteins for the occurrence of patterns, such as the CxxCH motif, typically found in *c*-type cytochromes (//pbil.ibcp.fr/cgi-bin/pattern_prosite).

6. Prediction of the protein localisation, by signal sequence analysis (www.cbs.dtu.dk/services/SignalP/).

Considering all the information provided by each step, the proteins were classified according to the heme numbers and cellular localization. Based on homology with known enzymes, functions were predicted for some of the proteins.

3.2.1 Protein classification based on the number of the hemes

Table 3.1 Classification of the identified *Shewanella* MR-1 proteins based on heme numbers

monohemes	cytc1, ccoO, shp208, cyc260B, c_4A333, c_4B333
dihemes	cyc32, $c_4$39, ccoP, ccp111, cyc133, cyc142, napB, $c_4$185, cyc185, cyc208, cyc220B
trihemes	$c_3$191
tetrahemes	fccB54, fccB56, fccB63, fccB342, c3MR-1, cymA, cyc220a, fcc_3, fcc_2MR-1
pentahemes	nrfA, torC
heptahemes	cyc202
octahemes	cyc260a
decahemes	cyc18, cyc129, omcA, mtrA, mtrC, mtrD, mtrE, mtrF

Few non-cytochrome redox proteins were identified: fccA54, fccA56, fccA63, fccA342, frdA, frdB, frdC, $frdC_2$, hal56, hal63.

3.2.2 Classification based on signal sequence

a. Bacterial proteins destined for export to the periplasm possess an N-terminal signal sequence which directs translocation across the cytoplasmic membrane by the Sec system. The bacterial signal sequences have little sequence conservation but have similar structural features and contain three distinct regions: a basic N-terminus (n) region, a central hydrophobic (h) region and a more polar C-terminus (c) region. Based

Table 3.2 Soluble proteins with typical signal sequence. The peptidase recognition sites are given in bold

Name	Signal sequence
Cyc260b	MMKTSTKIAIAAALMGCLATQ**AYA**
$c_4$185	MNNKIKTLAKGIALISGCALTSV**AMA**
$c_4$39	MKKLALALSVVVAAISSP**AIA**
c4a333	MLLRAIYSAGVLMLLLPAV**THA**
c_4b333	MKTFIVSLLVLCGLSTA**VYA**
Ccp111	MTKLTAIAITLTAIFASSY**AVA**
fcc_3	MFTRKIQKTALAMLISGAMAGT**AYA**
Fcc_2MR-1	MLNTKLLPLAMSAFFISTF**SYG**
FccA342	MIYLKIIRGFFRALVPFS**ANA**
Cyc260a	MKQLLFIALAGMALQ**AQA**
Shp208	MNHHLNKHRLELAITAVLLASFNAFA**ANA**
hal56	MKLKTIPLVIAAILPMT**SIA**
hal63	MLEIIMNKSVLMASLTLSLLSGS**ALA**
Cyc208	MTKPTHTLSWIAGSTLTALALTIGIIGISLTFSST**GYA**
NapB	MKKILTLAAIVLAIGGCSG**QQA**
$c_3$191	MARILLVFLSCLLSNV**VYA**
Cyc202	MMKRWKTKTALGVLFCLGS**AVS**
MtrA	MKNCLKMKNLLPALTITMAMSAVMALVVTPN**AYA**
MtrD	MDIGLKFNSITQIMLTLMLSILSLS**TLA**
FccB54	MMKTLLLTLVVATLFTGA**ANA**
FccB56	MMKYIYKLGLVSALLMTCLS**AYS**
FccB63	MLKPVFILLLGFMLATPMA**ANA**
FccB342	MKLMNTLIVIALSSFIALS**VHA**
NrfA	MSCFTTQMLRGDEMMKKMTGKTFALSALVAASFMAAG**AMA**
Cyc133	MMSIN**RR**QA**L**TRIIGISTAAAGATLMGTS**AFA**

on statistics, von Heijne (1986) suggested that a signal sequence for a periplasmically located protein would present the following features:

M-2-15 basic residues-α **helix-(P / G)-X-X-(A / G / S)-X-(A / G / S)** ↓ anything but Pro

These leader peptides are removed at a later stage in the export process by a signal peptidase (Pugsley, 1993). The proteins identified in *Shewanella* MR-1, with a typical signal peptide, are shown in Table 3.2.

b. Soluble proteins with a double arginine signal sequence

There is a group of proteins that contain an unusually long signal sequence (26-58 amino acids in length) with a consensus motif in which the arginine residues are invariant (Berks, 1996).

(S / T)-R-R-X-F-L-K

The signal peptidase processing a double arginine signal sequence has the same recognition site as in the Sec pathway (probably the same enzyme operates for both pathways; Berks *et al.*,2000). These proteins bind redox cofactors and are exported in a Sec-independent manner. The cofactors associated are: Fe-S clusters, molybdopterin cofactor, certain polynuclear copper sites and the FAD. The acquisition of the cofactor is essential for the translocation. The proteins cross the cytoplasmic membrane in a stable, folded conformation, with their metallocentre functionally formed (Berks *et al.,* 2000). Proteins with a double arginine signal sequence are found in Table 3.3.

Table 3.3 Soluble proteins with a double arginine signal sequence

Name	Signal sequence
fccA54	MHD**RRSFLK**LGAGAAVGGIAAALPSA**ASA**
fccA56	MAG**RR**D**FLK**LATGITA**AMA**
fccA63	MSKTQDINAG**RR**QL**LK**GGMVMAAGLGASMALP**ATA**

c. Lipoprotein signal sequence

Statistically, membrane-bound proteins present a hydrophobic signal peptide with the following sequence:

M- 2-15 basic residues **-α helix-L-X-(A / G)** ↓ **C-D**

The amino-terminal cysteine of the proteins listed in Table 3.4 must be modified to glycerylcysteine for cleavage to occur (Pugsley, 1993).

d. Membrane-bound proteins

Some proteins are integral membrane proteins that have one or more stretches of hydrophobic amino acids in the lipid bilayer: cyc 32, omcA, mtrC, ccoP, ccoO, torC, frdC and $frdC_2$.

Table 3.4 Lipoproteins (the peptidase recognition site is given in bold)

Name	Signal sequence
cyc129	MMKNYNKSLLALALTSALC**LTA** CG
mtrF	MNKFASFTTQYSLMLLIATL**LSA** CG

3.3 Decahemes and outer membrane cytochromes

As mentioned in the introduction, *Shewanella* spp are among the few organisms able to generate energy via electron transport linked to the reduction of manganese (IV) and iron (III) oxides. Considering that these oxides are insoluble at physiological pH, the bacterium faces an interesting problem: how can it transform insoluble oxides into soluble, accessible substrates? To overcome this it was speculated that the cell would need to make close contact with the metal oxides and to excrete solubilization factors outside the cell or to locate terminal reductases on the outer membrane. Recently it has been shown that cell contact is not a requisite for the reduction of solid Fe(III) oxides: an adhesion deficient *S. alga* strain can reduce amorphous Fe(III) oxides at the same rates as the strain that strongly adheres to Fe(III) oxides (Gaspard *et al.*, 1998). To date, no extracellular reductases have been isolated from *S. putrefaciens*
MR-1, but it was shown that the vast majority of membrane-bound *c*-type cytochromes are found in the outer membrane of anaerobically grown cells (Myers & Myers, 1992a) where they could potentially make direct contact with the insoluble metal oxides. This novel distribution is in contrast to that of other bacteria in which cytochromes are typically located in the cytoplasmic membrane and the periplasm.

From genome analysis, eight decaheme cytochromes were identified: Cyc18, Cyc129, MtrA, MtrC, MtrD, MtrE, MtrF and OmcA. OmcA and MtrC have been shown to be outer membrane proteins (Myers & Myers, 1998, Beliaev *et al.,* 2001); Cyc129, and MtrF have a lipoprotein signal sequence which indicates that they are membrane anchored (see Table 3.4); MtrA is periplasmically located (Beliaev *et al.,* 2001) and the

signal peptide of MtrD indicates the same location. Cyc18 exhibits one or more transmembrane helices.

Six of these genes are organised into a 13 kb cluster *mtrDEF-omcA-mtrCAB* (GeneBank accession number AF083240), with only one promoter, upstream of *mtrC*, indicating that *mtrCAB* form an operon (Beliaev *et al.*, 2001). MtrA and MtrB seem to be part of the electron transport chain that leads to Fe(III) reduction (Beliaev & Saffarini, 1998), but are not involved in the final step (Beliaev *et al.*, 2001); mutants that lack either MtrA, MtrB or both are deficient in Fe(III) reduction *in vivo*, but this deficiency was not detected when the membrane fractions were used to measure Fe(III) reductase activity *in vitro*. Mutants that lack the outer membrane MtrC exhibit low levels of Fe(III) reduction, *in vivo* and *in vitro*, compared with the wild-type strain, suggesting that it may play a role in the final step of Fe(III) reduction or may be required for the assembly or stability of the reductase (Beliaev *et al.*, 2001). It is speculated that *mtrDEF* encodes a second, lower activity, ferric reductase, responsible for the residual ferric reductase activity measured in the ΔMtrCAB strain (Beliaev *et al.,* 2001). In all the mutant strains, the Fe(III) phenotype was accompanied by a Mn(IV) deficiency as well.

There is a second, contradictory report regarding the role of MtrC. Myers & Myers reported that upon inactivation of *mtrC* and *omcA* the cells are unable to metabolise Mn(IV), but the Fe(III) phenotype is comparable to the wild type (Myers & Myers, 2001). It is difficult to understand these results, especially when the genes were

inactivated in similar manners in both reports and Fe(III) reductase activities were measured by the same method.

The function of the other two decaheme cytochromes, Cyc18 and Cyc129 is unknown. Although membrane-bound, Cyc129 is not involved in iron or manganese reduction. Insertional inactivation of the corresponding gene expressed the same phenotype as the wild-type strain (see Chapter VI). Considering the homology with the MtrA and MtrD proteins (52 and 46 % identity respectively), Cyc18 may function as an electron shuttle between the tetraheme CymA (see Chapter 3.6) and a terminal iron reductase.

3.4 Fumarate reductases

Fumarate reductase catalyses the reduction of fumarate to succinate (see Chapter 1.3.2.2; flavocytochrome c_3, Fcc_3) and it has been isolated from *S. frigidimarina* NCIMB400 and *S. putrefaciens* MR-1. The enzyme is periplasmically located, essentially unidirectional and contains three domains: the N-terminal cytochrome domain, with four covalently-bound *c*-type heme groups, the FAD binding domain, which contains non-covalently bound FAD and the acitve site, and the clamp domain, which is likely to be involved in controlling access of the substrate to the active site (Taylor *et al.,* 1999). The *Shewanella* fumarate reductase is induced during anaerobiosis in the presence of fumarate (Pealing *et al.,* 1992) and is required for fumarate respiration, as demonstrated by gene inactivation (Gordon *et al.,* 1998; Myers & Myers, 1997a). However, a second fumarate reductase has been isolated from *S. frigidimarina*

```
fcc3_MR1         MFTRKIQKTALAMLISGAMAGTAYAAPEVLADFHGEMGG-CDSCHVSDKGGVTNDNLTHE 59
fcc3_NCIMB400    MKKMNLAVCIATLMGTAGLMGTAVAA-DNLAEFHVQNQE-CDSCHTPD-GELSNDSLTYE 57
ifc3_NCIMB400    ---MKLKYLVSAMALVVLSSGTAMAKTPDMGSFHADMGS-CQSCHAKP--IKVTDSETHE 54
fcc2_MR1         ----MLNTKLLPLAMSAFFISTFSYG-QSLSEFHIDKGNNCQTCHTKP--IKVDDAENHE 53
                     :     .:        .*        :..** :     *::**.        *  .:*

fcc3_MR1         NGQCVSCHGDLEELAAAAPKDKVSPHKSHLIGEIACTSCHKGHEKSVAYCDACH-SFGFD 118
fcc3_NCIMB400    NTQCVSCHGTLAEVAETTKHEHYNAHASHFPGEVACTSCHSAHEKSMVYCDSCH-SFDFN 116
ifc3_NCIMB400    NAQCKSCHGEYAELAN--DKLQFDPHNSHLG-DINCTSCHKGHEEPKFYCNECH-SFDIK 110
fcc2_MR1         NKSCESCHGTMGELAAK-SKSKLSPHHSHLI-DVSCTSCHSGHKQPVFVCQTCHDSFKNE 111
                 * .* ****    *:*     : : ..* **:  :: *****..*::.   *: ** **  .

fcc3_MR1         --MPFGGKWER---KFVPVDADKAAQDKAIAAGVKETTDVVIIGSGGAGLAAAVSARDAG 173
fcc3_NCIMB400    --MPYAKKWLRDEPTIAELAKDKSERQAALASAPHDTVDVVVVGSGGAGFSAAISATDSG 174
ifc3_NCIMB400    P-MPFSDAKKK---KSWDDGWDQDKIQKAIAAGPSETTQVLVVGAGSAGFNASLAAKKAG 166
fcc2_MR1         FKIPFSNDKPVLD-NYQFPEVTQEMIETALAKAPLEQHQVIVIGAGAAGHAAAISARQHG 170
                   :*:.        .         :    : *:* .  :   :*:::*:*.**  *:::* . *

fcc3_MR1         -AKVILLEKEPIPGGNTKLAAGGMNAAETKPQAKLGIEDKKQIMIDDTMKGGRNINDPEL 232
fcc3_NCIMB400    -AKVILIEKEPVIGGNAKLAAGGMNAAWTDQQKAKKITDSPELMFEDTMKGGQNINDPAL 233
ifc3_NCIMB400    -ANVILVDKAPFSGGNSMISAGGMNAVGTKQQTAHGVEDKVEWFIEDAMKGGRQQNDIKL 225
fcc2_MR1         VADVVILEKQPYIGGNSMLAAGGMSAAETVTQALKHYPDSKALWYEDTMKGGHNINNPDL 230
                   *.*::::* *   ***: ::****.*. *  *        *.      :*:****:: *:  *

fcc3_MR1         VKVLANNSSDSIDWLTSMGADMTDVGRMGGASVNRSHRPTGGAGVGAHVSQVLWDNAVKR 292
fcc3_NCIMB400    VKVLSSHSKDSVDWMTAMGADLTDVGMMGGASVNRAHRPTGGAGVGAHVVQVLYDNAVKR 293
ifc3_NCIMB400    VTILAEQSADGVQWLESLGANLDDLKRSGGARVDRTHRPHGGKSSGPEIIDTLRKAAKEQ 285
fcc2_MR1         VKILTENGSAGVDWLQAMGADLTSASSAGGHNAERLHRPTGGAKSGPEIVNTLKETAQKL 290
                 *.:*:.:.   .::*: ::**:: .      **   .:* *** **    *..: :.* . * :

fcc3_MR1         GTDIRLNSRVVRILEDASGKVTGVLVKGEYTGYYVIKADAVVIAAGGFAKNNERVSKYDP 352
fcc3_NCIMB400    NIDLRMNTRGIEVLKDDKGTVKGILVKGMYKGYYWVKADAVILATGGFAKNNERVAKLDP 353
ifc3_NCIMB400    GIDTRLNSRVVKLVVNDDHSVVGAVVHGKHTGYYMIGAKSVVLATGGYGMNKEMIAYYRP 345
fcc2_MR1         GVETRTNSKVIQLVQNKQGVITGVLVQGKHSKLHILGAKAVIIASGGFARNNELVAKYRP 350
                 . : * *:: :.:: : .   : * :*:* :.   : : *.:*::*:**:. *:* ::   *

fcc3_MR1         KLKGFKATNHPGATGDGLDVALQAGAATRDLEYIQAHPTYSPAGGVMITEAVRGNGAIVV 412
fcc3_NCIMB400    SLKGFISTNQPGAVGDGLDVAENAGGALKDMQYIQAHPTLSVKGGVMVTEAVRGNGAILV 413
ifc3_NCIMB400    TMKDMTSSNNITATGDGVLMAKEIGASMTDIDWVQAHPTVGKDSRILISETVRGVGAVMV 405
fcc2_MR1         DLKGVDATNNPGNVGDALTFAPKVGADIVDVKEIQAFPTAA-AGKMVISGTARGAGAIML 409
                   :*.. ::*:     .**.: .* : *.    *:. :**.** .   . :::: :.** **:::

fcc3_MR1         NREGNRFMNEITTRDKASAAILQQKGESAYLVFDDSIRKSLKAIEGYVHLNIVKEGKTIE 472
fcc3_NCIMB400    NREGKRFVNEITTRDKASAAILAQTGKSAYLIFDDSVRKSLSKIDKYIGLGVAPTADSLV 473
ifc3_NCIMB400    NKDGNRFISELTTRDKASDAILKQPGQFAWIIFDNQLYKKAKMVRGYDHLEMLYKGDTVE 465
fcc2_MR1         NNDGERFCDEMGPRDKVSECIWAQKGKDAWLVFDETVLDRLGQLRGMLDLGIIFKGNSAD 469
                 *.:*:** .*: .***.* .*  * *: *:::**: : .      :      * :    ..:

fcc3_MR1         ELAKQIDVPAAELAKTVTAYNGFVKSGKDAQFERPDLPRELVVAPFYALEIAPAVHHTMG 532
fcc3_NCIMB400    KLGKMEGIDGKALTETVARYNSLVSSGKDTDFERPNLPRALNEGNYYAIEVTPGVHHTMG 533
ifc3_NCIMB400    QLAKSTGMKVADLAKTVSDYNGYVASGKDTAFGRADMPLNMTQSPYYAVKVAPGIHHTMG 525
fcc2_MR1         EMAKATKMNAQKFDQAIKRYNDFKVKGKDADFARKNMADDL-QYPIYAVKIKPAVHHTMG 528
                 ::.*   :     : :::  **.    .***: * * ::.  :       **::: *.:*****

fcc3_MR1         GLVIDTKAEVKSEKTGKPITGLYAAGEVTGGVHGANRLGGNAISDIVTYGRIAGASAAKF 592
fcc3_NCIMB400    GVMIDTKAEVMNAKK-QVIPGLYGAGEVTGGVHGANRLGGNAISDIITFGRLAGEEAAKY 592
ifc3_NCIMB400    GVAINTTASVLDLQS-KPIDGLFAAGEVTGGVHGYNRLGGNAIADTVVFGRIAGDNAAKH 584
fcc2_MR1         GLKINTKTEVIDQNG-LPIKGLFAAGEVTGGVHGANRLAGNAIADTIVFGRIAGEQVANS 587
                 *: *:*.:.* . :     * **:.********** ***.****:* :.:**:** ..*:
```

Fig 3.1 Amino acid sequence alignments of fumarate-reductases from *Shewanella* MR-1 and *S. frigidimarina* NCIMB400 (heme binding sites and the conserved histidines are shown in red)

NCIMB400 (Dobbin *et al.*, 1999). This enzyme is a soluble tetraheme flavocytochrome c_3 (ifc_3), induced only during the respiration with Fe(III) citrate. It is very similar to Fcc_3 in its amino acid sequence and behaviour of redox centres, but it is not synthesised during fumarate respiration and nor is it essential for iron(III) respiration. From sequence analysis of the *Shewanella* MR-1 genome, a second, soluble (see Table 3.2) fumarate reductase has also been identified (Fcc_2-MR1), which is 41 % identical to ifc_3 from *S. frigidimarina* NCIMB400 and 42 % identical with Fcc_3 from *Shewanella* MR-1 and NCIMB400. This enzyme presumably also possess a unidirectional fumarate reductase activity, like ifc_3 (Dobbin *et al.,* 1999; see Fig 3.1) but its physiological role is unknown.

Shewanella spp are the only bacteria known to produce soluble respiratory fumarate reductases, whose roles have been elucidated by using null mutation construction (Gordon *et al.,* 1998) and transposon mutagenesis (Myers & Myers, 1997a). However, it appears that the genome of *Shewanella* MR-1 contains an operon that is very similar to the *Wolinella succinogenes* fumarate reductase operon (*frdCAB*), which encodes a membrane-bound enzyme. FrdA lacks a signal sequence, is 60 % identical to the FrdA subunit of the *W. succinogenes* fumarate reductase and is predicted to bind FAD covalently. FrdB is 56 % identical to the *Wolinella* FrdB subunit and contains one [2Fe-2S] ($C_{59}C_{64}C_{67}C_{79}$), one [3Fe-4S] ($C_{164}C_{211}C_{217}$) and one [4Fe-4S] signature ($C_{154}C_{157}C_{160}C_{165}$), as predicted by the "Prosite Scan" programme and by comparison to the *Wolinella* subunit. FrdC is predicted to be a membrane-bound *b*-type cytochrome with His_{93} and His_{182} as proximal ligands and His_{44} and His_{143} as the distal ligands. FrdC in *Wolinella* anchors the enzyme to the membrane (Lancaster *et al.*, 1999) and is

essential for the enzyme stability (Simon *et al.*, 1998a). Downstream of the *frdCAB* operon there is a second *frdC* gene (*frdC*$_2$) which is predicted to encode for another cytochrome *b*. A similar arrangement appears in *Wollinella*, but this second *b*-type cytochrome is not found in the isolated enzyme and is not required for fumarate respiration (Simon *et al.*, 1998a; Lancaster *et al.*, 1999; see Fig 3.2).

The reason for the presence of three fumarate reductase proteins in *Shewanella* MR-1 is unknown, as only one enzyme is required for fumarate respiration. Four more fumarate reductase-like proteins were found, two of them with a rather unusual organisation. These four putative flavocytochromes are about 40 % identical to Fcc$_3$,

```
frdC_MR1            ------MAIKLSIK------KWSAWLDLSQSVSGVILAVFLWTHLVLVSSILLGGDAMHW 48
frdC2_MR1           --MISVTRVHTRVKTQGIGHPWSAKADRLQSATGIMLGCFLLLHMHFESSILLGKEAFYQ 58
frdC_W.succin       MTNESILESYSGVTPERKKSRMPAKLDWWQSATGLFLGLFMIGHMFFVSTILLGDNVMLW 60
frdC2_W.succin      ----MKNVTTFDARCCKRKSRMPAKLDRWQSLTGLFLALFMTCHMIFTSTILLGPEAFDW 56
                                          .*  *  ** :*::*. *:  *: : *:**** :.:

frdC_MR1            VARTMELSFLSSDGRGFPWVVTCIAIGIAALALVHVLVALQKLPMSLRQQRALQQQMQVI 108
frdC2_MR1           VVQLLEGGMFSSTGHGFPMVTKVFSVFMLLVVTLHAAVALRRFPAQLGQWRALRSHLGCT 118
frdC_W.succin       VTKKFELDFIFEGGK--PIVVSFLAAFVFAVFIAHAFLAMRKFPINYRQYLTFKTHKDLM 118
frdC2_W.succin      VIGKAEMDFWFEGGL--PWVTSLVTLGVLVLFLAHGFLAMRKLPANYLQLAQFLSHRQLL 114
                    *    * .:  . *   * *.. .:  :  : : *  :*::::* .  *   :  :   :

frdC_MR1            NHSDTRLWRWQVITGVVILLLLPVHLWLIGSAPETIGPQGSADRIWNQGVWMVYLPLLLT 168
frdC2_MR1           NHRDTHAWFWQLITGFILFFLVPVHLFTMILNPE-IGPHLSAERVYHDNAWLLYALLLPA 177
frdC_W.succin       RHGDTTLWWIQAMTGFAMFFLGSVHLYIMMTQPQTIGPVSSSFRMVSEWMWPLYLVLLFA 178
frdC2_W.succin      SHTGTTLWWLQCLSGLALLVLGGAHLLMILTSKETLSATTAAYRFVHGGLGSFYLLLLLV 174
                     * .*  *  * ::*. ::.*  .**  :    : :..  :: *.       .*  ** .

frdC_MR1            VELHAAIGIYRVALKWG---AALDLNSRSRLRKIKTIVSVAFVTVGLASLLAFLPYAS-- 223
frdC2_MR1           VVIHAMIGLYRVAVKWG---ITAQ---RSGLRKLAKVLIIYLLCLGSASLVAYMLIGSEL 231
frdC_W.succin       VELHGSVGLYRLAVKWGWFDGETPDKTRANLKKLKTLMSAFLIVLGLLTFGAYVKKGLEQ 238
frdC2_W.succin      STLHAGIGAYRLILKWCPIEASEPKTLQRRIRNVRSEVFGVFGVLTLLALWADFTYIKHG 234
                      :*. :* **: :**            :  :::: . :   :  :  :: * .

frdC_MR1            ------------------
frdC2_MR1           SIPVQPYVPQ-------- 241
frdC_W.succin       TDPNIDYKYFDYKRTHHR 256
frdC2_W.succin      KNLSPLSIQERLTFAKTK 252
```

Fig 3.2 Amino acid sequence alignments of FrdC and FrdC2 from *W. succinogenes* and *Shewanella* MR-1

but whereas Fcc_3 is a single polypeptide with distinct cytochrome and flavoprotein domains, each of these novel proteins is apparently composed of two separate polypeptides: a flavoprotein (FccA) and a tetraheme *c*-type cytochrome (FccB), since they are closely linked in a putative operon. In two cases, a third reading frame (Hal) is closely associated, which encodes a homologue of histidine ammonia lyase (see Fig 3.3).

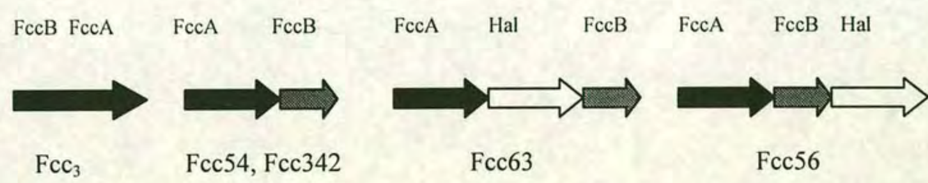

Fig 3.3 Organisation of the Fcc54, Fcc56, Fcc63 and Fcc342 genes

FccA proteins are soluble and with the exception of FccA342, all have a double arginine signal sequence (see Table 3.3) which ensures Sec-independent Tat-mediated transport to the periplasm (see Chapter 3.2.2). The FccB subunits are soluble too, as indicated by the signal sequences (see Table 3.2). They are small, tetraheme *c*-type cytochromes (94-105 amino acids) with two apparent domains, each containing two heme binding sites separated by 9-12 amino acids. The Hal subunits (in Fcc56 and Fcc63) are also soluble (see Table 3.2) and resemble the histidine ammonia lyase which catalyses the first step in histidine degradation (Schwede *et al.*, 1999; see Fig 3.4). The significance of this organisation is unknown.

Sequence alignment and secondary structure prediction were used for molecular modelling to predict the three dimensional structure of the catalytic domain, using the known active site structure of Fcc_3 as a molecular framework. Some of the amino acids

involved in fumarate binding in Fcc_3 are not conserved in Fcc54, Fcc56, Fcc63 and Fcc342 and the active site might accommodate a monocarboxylate substrate rather than a dicarboxylate, substrates that are widespread in nature as degradation products of organic material (Bilsland, personal communication).

```
Hal56_MR1           RIRASYKAPIDAAEDGRSIYGLTVNYGALKDQRVAGAS-VENEPNRSESIKFNERQMRIQ 112
Hal63_MR1           QLVKAHELLMEAARLGKPVYGLTVGVGLNKDHKLFDANGKLSDAVLDASRSFNFSTLRAH 119
Hal_P.putida        AIDASVACVEQIIAEDRTAYGINTGFGLLASTRIASHD----------LENLQRSLVLSH 84
                     :  :     :    .:. **:... *   . :: . .            .::   :  :

Hal56_MR1           AAGMEPFLPEAESKMAMVIRLNQMAAGFTGMSVDAANAYMEYINNDVYPLIPSRGSEGAN 172
Hal63_MR1           SAGVGEAAPIRLTRVALAVRLNTMLAGQTGVQPEVAELYEAYLNKGLTPVIPSKGTVGEA 179
Hal_P.putida        AAGIGAPLDDDLVRLIMVLKINSLSRGFSGIRRKVIDALIALVNAEVYPHIPLKGSVGAS 144
                    :**:         :: :.:::* :  * :*:  .. :     :*  : * ** :*: *

Hal56_MR1           -DLSMVTHVGLALMGEWDVMYQGKRRSSAEVRKELGLKKYQPFGMDGISILSNSNVAEAQ 231
Hal63_MR1           -DILLSSHVGLAMIGEWEVFYKGQRVSSKEAMADAGITPLVPMGKDALSILSNNAFAVAY 238
Hal_P.putida        GDLAPLATMSLVLLGEGKARYKGQWLSATEALAVAGLEPLTLAAKEGLALLNGTQASTAY 204
                     *:   : :.*.::** .. *:*: *: *.     *:      . :.:::*...  : *

Hal56_MR1           SITAVKKVEHLLDLSSVIIASSLEALNGNVSPFLWHTVDTKGWPQGHEAAESILSHLKGS 291
Hal63_MR1           AMQGYREAKQLLSVSPTVFGLSLEGLNGNVAPFLPQTNDIRPFPYIKTATSDILKQLDGS 298
Hal_P.putida        ALRGLFYAEDLYAAAIACGGLSVEAVLGSRSPFDARIHEARGQRGQIDTAACFRDLLGDS 264
                    :: .   .:.*   : .  . *:*.: *. :**  :  : :       ::  : . * .*

Hal56_MR1           YLWSLDSK--RNLQDPLSFRSSGQILAAAKEELRQAKELLNTAINHTTDNPIVHTNARDD 349
Hal63_MR1           YLWQLNDE--RPLQDPLSFRTTAYTFAGAEQALASLDEVINIQINHSDDNPAVIVGASN- 355
Hal_P.putida        SEVSLSHKNCDKVQDPYSLRCQPQVMGACLTQLRQAAEVLGIEANAVSDNPLVFAAEGD- 323
                       .*. :    :*** *:*     :...   * .  *::.   *   *** * .   :

Hal56_MR1           LWYSNSDYVDGLRVGGKNVFVNSGSNFDNTQLAVQLESLSRALAQVIHISAWRTTQLDDG 409
Hal63_MR1           -QYAQFPQVAKYVVEGQGGVFPT-TNFEPLPVALAVQNLSVALTHVSHNSVMRTIHLSDE 413
Hal_P.putida        --------------------VISGGNFHAEPVAMAADNLALAIAEIGSLSERRISLMMDK 363
                                        . :  **.   :*:  :.*: *::.:   *  *   : *

Hal56_MR1           HRTKLTTYLISKENVGGDGFANIAQSMSGLYAEAMSLTNSAAIYGVPTSVGIEETFSNVN 469
Hal63_MR1           HFTKLTRFLSAPENQG-HAFGAIQKAFVDMQVRNKQLATPVSFDGISIAGGIEDTFTNLK 472
Hal_P.putida        HMSQLPPFLVENGGVN-SGFMIAQVTAAALASENKALSHPHSVDSLPTSANQEDHVSMAP 422
                    * ::*. :*    . .  .*     :   :  .   *: . :. .:. : . *: .:

Hal56_MR1           LIANRLDKIADIAYEIYSYEVLHTTQGMDIRTKEY-SQKMGNGTSEFLHEYRKYVPFVSK 528
Hal63_MR1           LASDNLIQIVDNTRVIYGLELLHSTQAIDLRKQANPELQLGKATQAMYKAYRAKVPFVAK 532
Hal_P.putida        AAGKRLWEMAENTRGVPAIEWLGACQGLDLRKGLKTSAKLEKAR----QALRSEVAHYDR 478
                      ...* ::.: :  : . * * : *.:*:*.    . :: :.     :  *  *..  :

Hal56_MR1           DRIYTPDINNGVKFLK 544
Hal63_MR1           DRPFTPDIQASTDFIT 550
Hal_P.putida        DRFFAPDIEKAVELLA 494
                    ** ::***: ...::
```

Fig 3.4 Amino acid sequence alignments of histidine ammonia lyase from *P. putida* and Hal56 and Hal63 subunits from *Shewanella* MR-1

An enzyme with similar organisation was isolated from *Geobacter sulfurreducens*. Its activity is dependent on the presence of both the flavoprotein and the cytochrome *c*. The two subunits are periplasmically located, tightly associated with each other and catalyse the hydrogenation of α-unsaturated monocarboxylic acids like methacrylate, acrylate, crotonate or pentenoate (Mikoulinskaia *et al.*, 1999).

In *W. succinogenes*, a flavoprotein (FccA) similar to the flavoprotein domain of Fcc_3 of *Shewanella* spp is specifically induced when the bacterium is grown with fumarate and sulfide, but it does not catalyse fumarate reduction by viologen radicals (Simon *et al.*, 1998b). Downstream, there are two tetraheme *c*-type cytochromes, FccB and FccC. FccB is soluble and FccC has a predicted N-terminal hydrophobic helix serving as a membrane anchor and resembles CymA (Simon *et al.*, 1998b). One of these proteins (FccA56) will be discussed in more detail in Chapters V and VI.

3.5 Cytochromes c_3

Cytochromes c_3 belong to the class III of cytochromes, according to Ambler's classification (Ambler, 1991). They are small proteins, with multiple bis-histidine ligated hemes and highly electronegative midpoint potentials.

By sequence analysis, two c_3-type cytochromes were identified in *Shewanella* MR-1: c_3MR-1 and $c_3$191. Cytochrome $c_3$191 is a soluble protein as indicated by the signal sequence (see Table 3.2), with 3 heme binding sites, the first one situated very close to the N-terminus (three amino acids distance). There are four histidine residues which may function as the sixth ligand (besides the ones involved in the CxxCH motif),

three of them situated between heme I and II and the fourth one near heme III. A triheme cytochrome (c_7) was isolated from *Desulfuromonas acetoxidans*. The three hemes present the same orientation as 3 of the 4 hemes of cytochrome c_3 (Aubert et al., 1998) and the protein was shown to be involved in the reduction of Fe(III). As the similarity with cytochrome c_7 from *D. acetoxidans* is weak (see Fig 3.5), no function can be predicted.

```
c3_191                MARILLVFLS----CLLSNVVYAID-GCNGCHDEKWQSTPAHIWIETEHHIDIVACPDCH 55
c7_D.acetoxidans      --ADVVTYENKKGNVTFDHKAHAEKLGCDACHEG----TPAKIAID-KKSAHKDACKTCH 53
                          ::.: .      :.: .:* . **:.**:    ***:* *: ::  .  **  **

c3_191                QWKEGDDLPPVLPSQETLQKECGQCHATPDNLPAMMHMQQYQQLK 100
c7_D.acetoxidans      KSNNG-------PT------KCGGCHIK----------------- 68
                      : ::*       *:      :** ** .
```

Fig 3.5 Sequence alignment of cytochromes c_7 from *D. acetoxidans* and $c_3$191

The second protein is c_3MR-1, a soluble cytochrome isolated by Tsapin *et al.* (1996) which contains four bis-histidine ligated, low potential hemes and the reduced form is rapidly oxidised by Fe(III) citrate . The protein exhibits 69 % identity with another tetraheme cytochrome isolated from *S. frigidimarina* NCIMB400, cytochrome c_3 (see Fig. 3.6)

```
c3_S.frigidimarina    ADETLAEFHVEMCCCENCHADCEPSKDCAYEFEQCQSCHCSLAEMDDNHKPHDCL 80
c3_MR1                ADQKLSDFHAESGGCESCHKDGTPSADGAFEFAQCQSCHGKLSEMDAVHKPHDGN 55
                      **:.*::**.* ****.** ** ** ***:** *******.*:***  ******

c3_S.frigidimarina    LMCADCHAPHEAKVGEKPTCDTCHDDGRTA 110
c3_MR1                LVCADCHAVHDMNVGQKPTCESCHDDGRTS 85
                      *:****** *: :**:****::*******:
```

Fig 3.6 Amino acid sequence alignments of cytochromes $c_3$195 from *Shewanella* MR-1 and c_3 from *S. frigidimarina* NCIMB400 (the heme binding sites and the conserved histidines are shown in red)

This cytochrome was shown, by gene inactivation, to be involved in the electron transfer to an iron reductase (Gordon *et al.*, 2000). The genes that surround these proteins are different in the two strains: 3-hydroxybutyrate dehydrogenase and an assimilatory nitrate reductase in *S. frigidimarina* NCIMB400 (accession number AJ000006) and a hydrogenase cytochrome *b* subunit and a heat-shock protease in *Shewanella* MR-1. The sequence differences between the two proteins could reflect the distant relationship between the two *Shewanella* strains (Gordon *et al.,* 2000).

3.6 Members of the NapC/NirT family

NapC/NirT family comprises *c*-type cytochromes, involved in anaerobic pathways and usually located in the vicinity of genes encoding the catalytic subunits of periplasmic reductases. The proteins are membrane-bound and contain four low-spin, bis-histidine ligated hemes.

CymA is a membrane-bound tetraheme cytochrome identified first in *S. putrefaciens* MR-1. Gene inactivation by transposon mutagenesis or gene disruption results in an inability of the bacterium to grow on fumarate, nitrate, Fe(III) and Mn(IV), but it is not required for TMAO respiration (Myers & Myers, 1997a; Myers & Myers, 2000). CymA shares partial homology to other members of the NapC/NirT family: NapC from *P. denitrificans* and *P aeruginosa* (30 and 32 % identity respectively), TorC from *E. coli* and *S. massilia* (35 and 31 % respectively), DmsC from *R. sphaeroides* (30 %) and NirT from *P. stutzeri* (28 %). Spectroscopic studies revealed that the hemes are low spin (with potentials between +10 and –229 mV) and bis-histidine ligated (Field *et*

al., 2000). When in the fully reduced state, one of the four hemes may lose a histidine ligand provided by the polypeptide and become a high-spin complex (Field *et al.*, 2000).

```
nirT_P.stutzeri       MTDKDGNKQQKGG----ILALLRRPSTRYSLGGILIVGIVAGIVFWGGFNTALEATNTET 56
napC_T.pant           MGWIRASIRWIWGRVTWFWRVISRPSSFLSIGFLTLGGFICGVIFWGGFNTALEITNTEK 60
torC_MR1              MKWLIN-----------LWRTLNKPTKALTLGAVSISAFIMGIIFWGGFNTALEATNTEA 49
cymA_MR1              MN----------------WRALFKPSAKYSILALLVVGIVIGVVGYFATQQTLHATSTDA 44
                      *                    : :*:   ::  : : .:: *:: : . : :*. *.*:

nirT_P.stutzeri       FCISCHEMGDNVYPEYKETIHYANRTGVRATCPDCHVPRDWTHKMVRKVEASKELWGKIV 116
napC_T.pant           FCTSCHEMRDNVYQELMPTVHFSNRSGVRASCPDCHVPHEWTDKIARKMQASKEVWGKIF 120
torC_MR1              FCISCHSMESKPYQELQETVHWSNHSGVRATCPDCHVPHNWSRKIARKMEASHDVWGWLF 109
cymA_MR1              FCMSCHSN-HSLKNEVLASAHGGGKAGVTVQCQDCHLPHGPVDYLIKKIIVSKDLYGFLT 103
                      ** ***.   .   *   : * ..::** . * ***:*:     : :*: .*::::* :

nirT_P.stutzeri       GT-IDTAEKFEAKRLTLARREWARMRASDSRECRNCHS-LESMSSDMQKQRARKQHEMAR 174
napC_T.pant           GT-ISTREKFLEKRLELAKHEWARLKANDSLECRNCHA-AVAMDFTKQTRRAPQIHERYL 178
torC_MR1              NT-VNTPEKFEAKRLEMASREWKRFDRDNSLACKNCHN-YNSMKWESMSPLAQKQMKRAA 167
cymA_MR1              IDGFNTQAWLDENRKEQADKALAYFRGNDSANCQHCHTRIYENQPETMKPMAVRMHTNNF 163
                         ..*   :  :*  * :     :  .:*  *::**      .    .   * :

nirT_P.stutzeri       ED----NLTCIACHKGIAHHLPE 193
napC_T.pant           ISG---EKTCIDCHKGIAHQLPD 200
torC_MR1              EI----DQSCIDCHKGIAHHLPE 186
cymA_MR1              KKDPETRKTCVDCHKGVAHPYPK 186
                              :*: ****:** *.
```

Fig 3.7 Sequence alignments of NirT (*P. stutzeri*), NapC (*T. pantotropha*), TorC and CymA (*Shewanella* MR-1); heme binding sites and the conserved histidines are shown in red)

TorC

TorC is a pentaheme *c*-type cytochrome involved in electron transfer to TorA, the catalytic subunit of TMAO reductase (see Chapter 1.3.2.3). The protein identified in *Shewanella* MR-1 is 97 % identical to TorC from *S. massilia* (Dos Santos *et al.*, 1998) and is part of a *torCAD* operon, where TorA is the active subunit. TorA is soluble, with a double arginine signal sequence and a probable molybdenum binding site.

TorC is predicted to be membrane-bound, with five heme binding sites: four grouped in a NapC-like domain and the fifth one situated towards the C-terminus. TorD might be a cytoplasmic TorC chaperone (Pommiere *et al.,* 1998).

3.7 Cytochromes c_4

Cytochromes c_4 belong to the class I cytochromes (see Chapter 3.1). They have a single heme binding site near the N-terminal end of an ~100 residues long polypeptide.

In most cases, cytochromes c_4 are believed to participate in electron transfer to the terminal oxidases (Giudici-Orticoni *et al*., 2000). Flavocytochrome *c* from *Chromatium vinosum* contains a c_4 diheme and acts as a sulfide dehydrogenase (van Beeumen, 1991). In *Thiobacillus ferrooxidans*, a c_4-type diheme cytochrome represents the Fe-rusticyanin oxidoreductase and is the primary cellular oxidant of ferrous ions in the iron respiratory electron transport chain (Guidici-Orticoni *et al*., 2000). In *S. violacea*, a bacterium that grows in a high pressure environment, cytochrome c_4 may act as a bypass of the electron system to overcome the atmospheric pressure stress (Yamada *et al*., 2000).

In *S. putrefaciens* MR-1, four c_4 cytochromes (which exhibit 32-35 % identity with known c_4-type cytochromes) were identified: monohemes c_4a-333 and c_4b-333 and dihemes c_4 39 and $c_4$185. They are all soluble cytochromes, probably periplasmically located (see table 3.2). There is a 9-17 amino acid stretch at the N-terminus before the first heme binding site. Based on sequence alignments, most probably the sixth ligand

for all four proteins is a methionine: Met $_{81}$ (c_4b333), Met $_{74}$ (c_4a333), Met $_{81}$ and Met $_{184}$ for both $c_4$39 and $c_4$185 (see Fig 3.8).

```
c4_39               MKKLALALSVVVAAISSPAI---AEGNAEAGKTKIIVCSACHGMDGNSMIDMYPKLAGQH 57
c4_P.stutzeri       MNKVLVSLLLTLG-ITGMAH---AAGDAEAGQGKVAVCGACHGVDGNSPAPNFPKLAGQG 56
c4_185              MNNKIKTLAKGIALISGCALTSVAMANTSVIPPAGQICVSCHGVEGEGVEPLGPRLAGLS 60
c4_A333             ---MLLRAIYSAGVLMLLLP---AVTHANESNKLLNMCKACHGESGMSRFEHIPNIGWQN 54
c4_B333             MKTFIVSLLVLCGLSTAVYAEGYQVDIERGKALATAQCESCHGLAMIAMAKQFPNLRGQK 60
                                .                        * :***    .     *.:

c4_39               ATYLQKQTHDFRSAAQSGGKD--GRMDPTMSGMAMPLSDQDTLDTSAYFSTQ-------- 107
c4_P.stutzeri       ERYLLKQLQDIKAGSTPGAPEGVGRKVLEMTGMLDPLSDQDLEDIAAYFSSQ-------- 108
c4_185              KEYITTQLQHFQAG---------VRQNATMMPMAMTLQGDGIEQVASYFSAKSP--KTEV 109
c4_A333             SDYLLKQLRAFKAG---------TRQDPTMTKVAQLLSEADMQQMAEYFYLG----KDKM 101
c4_B333             PAYLNKQLVDFKAG---------LRLDPLMQSQVSALTAEQLQDVALFYSQQDIPNLSAN 111
                      *: .*:  :::.          *    *      *    : ::: ::

c4_39               KIQVAEVK-DVPELGAKLYKGG---DVSRG----ITACMACHGPDGKGAESAGFPALAGQ 159
c4_P.stutzeri       KGSVGYADPALAKQGEKLFRGG---KLDQG----MPACTGCHAPNGVGNDLAGFPKLGGQ 161
c_4185              KPVLRGTHVTFSDDTARLAYQG---DWSRG----LPACVTCHGPSGLGGDL--FPRLAGQ 160
c4_A333             LLRAIYSAGVLMLLLPAVTHAN---ESNKL----LNMCKACHGESG-MSRFEHIPNIGWQ 153
c4_B333             MKTFIVSLLVLCGLSTAVYAEGYQVDIERGKALATAQCESCHGLAMIAMAK-QFPNLRGQ 170
                          .      :   .   . .:        *  **.          :* :  *

c4_39               HANYTKLQLTKFRDAGRHNDLN-GMMQDVAKKLNDSDTDALSKYLASLK------- 207
c4_P.stutzeri       HAAYTAKQLTDFREGNRTNDGDTMIMRGVAAKLSNKDIEALSSYIQGLH------- 210
c4_185              QASYIKTQLLAWQAGTRKGDVD-GMMANVANKLTAAEVDALANYFANIK------- 208
c4_A333             NSDYLLKQLRAFKAGTRQ---D-PTMTKVAQLLSEADMQQMAEYFYLGKDK----- 200
c4_B333             KPAYLNKQLVDFKAGLRLD----PLMQSQVSALTAEQLQDVALFYSQQDIPNLSAN 222
                    :. *   **  :: . *        *   .  *.  ::: :: :    .
```

Fig 3.8 Amino acid sequence alignment of c_4-type cytochromes from *S. putrefaciens* MR-1 and *P. stutzeri* (c_4a333 and c_4b333 sequences were doubled; the heme binding sites and conserved methionines are shown in red)

The similarity between the two halves of the diheme c_4 cytochromes is quite high, indicating a gene duplication of a single small monoheme class I cytochrome gene. This is not an unusual feature for diheme c_4 cytochromes, and is found in *Azotobacter vinelandii* (Ambler *et al.*, 1984) and *Thiobacillus ferrooxidans* (Guici-Orticoni *et al.*, 2000).

3.8 Split-Soret diheme (Cyc133)

The "split-Soret" cytochrome was isolated from *Desulfovibrio desulfuricans* ATCC 27774, a sulfate-reducing, obligate anaerobe bacterium (Liu *et al.*, 1988). The protein is a dimer with two identical subunits. After reduction with sodium dithionite, the protein exhibits an absorbance maximum at 424 nm for the Soret band and a shoulder appears at 415 nm in the UV-visible spectrum. The physiological role is unknown: it has no significant nitrate or nitrite reductase activities and is present in both sulfate- and nitrate-grown cells. The heme groups are attached to one end of the protein and exposed to the solvent (Matias *et al.*, 1997). The sixth ligand is a histidine residue supplied by the other monomer, confirming that the functional unit is the dimer (Matias *et al.*, 1997).

Shewanella MR-1 appears to contains one "split-Soret" cytochrome. The presence of this cytochrome has not been detected in *W. succinogenes* or *E. coli* (Matias *et al.,* 1997) and constitutes a novel feature for *Shewanell*a MR-1. Cytochrome 133 is soluble, as indicated by the signal sequence (see Table 3.2) but it also presents a double arginine motif in the signal sequence which is unusual for a heme-binding protein. Analysis of the three dimensional structure of the "split-Soret" from *D. desulfuricans* showed that the protein can be regarded as being composed of two parts: the heme binding region and a domain which contains nine helices (Matias *et al.,* 1997) and four cysteine residues that are not involved in heme binding or disulfide bridges, but probably in binding of a [2Fe-2S] cluster, which was detected by spectral analysis (Devreese *et al.,* 1997).

Cyc133 shares few sequence similarities with the "split-Soret" cytochrome from *D. desulfuricans*:

-two heme binding sites situated towards the C-terminal end, separated by an 18 amino acid stretch (16 in *D. desulfuricans*), similar to the tetraheme cytochrome c_3 isolated from *Desulfovibrio* spp.

-four cysteine residues (same as in *D. desulfuricans*) which are not involved in heme binding and could bind a Fe-S cluster and / or another cofactor

-five histidines (versus 4) that could be the sixth ligand.

```
133_MR1                   LAYQ----TGGGCMHQVFHSVVTMLAQSNSADADKFKSIPTALAGYGFAGVTGQGTLCGN 116
dihm_D.desulfuric         VAYDGYWYKGFGCGFGAFYSIVGLMGEKYGAP---YNQFPFAMLEANKGGISDWGTIYGA 88
                          :**:    .* ** ..*:*:* ::.:.  .*    ::.:* *:  . .*::.  **: *

133_MR1                   LNAIGMLVNILDDINGQNNAVIANTFRYYENTTLPLTSPEFIAGIGSTAEKTALVGSSSK 176
dihm_D.desulfuric         LYGAAATFSLFWGRK-EVHPMVNELFRWYEVTKLPIFNP------GDAAQGVKGDLPMSA 141
                          * . .  ..:: . : : :.:: : **:** *.**: .*      *.:*: .    . *

133_MR1                   AMSVLCHSSISNWSRASGLPFS--KKAERCYRLSASMTYHLVELLN-RAYQGENIGALPG 233
dihm_D.desulfuric         SDSVLCHISVSKWCYENKIEATSKQRSERAGRLTADAAFKAAEIINTKIDQGKDFKSTFP 201
                          : ***** *:*:*.  . :  :  :::**. **:*. ::: .*::* :  **::: :

133_MR1                   SKPSLEAQDCQVCHGSSGTLGSAASVKTDMECTTCHTGHFN--------- 274
dihm_D.desulfuric         MQASVSS--CGECHMTKG--NDANWAKGIMDCTPCHSGTAATQNKFVNHP 247
                           :.*:.:  *  ** :.*  ..*  .*  *:**.**:*
```

Fig 3.9 Amino acid alignment between Cyc133 and the Split-Soret diheme from *D. desulfuricans* (heme binding sites are shown in red)

The data acquired for the "split-Soret" protein suggest that this cytochrome resulted from a gene fusion between a Fe-S protein and a diheme protein. This hypothesis might explain the presence of the double-arginine motif in the signal sequence of Cyc133. Fe-S clusters associated with a double arginine motif in the signal sequence have been identified in the soluble periplasmic nitrate reductase NapA from

Thiosphaera pantotropha (Berks *et al.,* 1995) and *Rhodobacter sphaeroides* (Reyes *et al.,* 1996).

3.9 Nitrate reductase

Nitrate reduction occurs in bacteria under anaerobic conditions (see Chapter 1.3.2.1). The periplasmic nitrate reductase is encoded in *E. coli* by the *napFDAGHBC* operon. In *Shewanella* MR-1 only *napDAGHB* genes were found. NapA is the soluble, catalytic subunit with a double arginine signal sequence and a molybdopterin binding site and is 64 % identical to the periplasmic nitrate reductase from *Pseudomonas* spp. NapB is a soluble protein (see Table 3.2) with two heme binding sites and most probably the sixth ligands are histidine residues (His_{72} and His $_{106}$). NapD, NapG and NapH have unknown functions.

3.10 Formate dependent nitrite reductase

In ammonification, nitrite is converted to ammonia by a nitrite reductase, in a pathway in which electrons are transferred from formate to the *c*-type cytochrome NrfA (cytochrome c_{552}). The enzyme has been isolated from *E. coli* (Darwin *et al.,* 1993), W. *succinogenes* (Simon *et al.,* 2000) and *Sulfurospirillum deleyianum* (Einsle *et al.,* 1999). NrfA is a soluble periplasmic protein (see Table 3.2) with four typical CxxCH and a rather unusual CxxCK heme binding sites. The crystal structure of NrfA from *S. deleyianum* shows that the physiological molecule is a homodimer in which all hemes, except heme 1, are bis-histidine coordinated (Einsle *et al.,* 1999). In *W. succinogenes* NrfA forms a complex with the product of the *nrfH* gene which allows fast electron

transfer between the formate dehydrogenase and the nitrite reductase (Simon *et al.*, 2000). This gene is absent in *Shewanella* MR-1.

3.11 Cytochrome *c* oxidase

Most of the bacterial oxidases are part of the superfamily of heme-copper respiratory oxidases. There are three classes: quinol oxidase, which contains heme and Cu_B (e.g. cytochrome bo_3 from *E. coli*) and cytochrome *c* oxidase which contains heme, Cu_A and Cu_B (e.g. cytochrome aa_3 from mitochondria); cytochrome *c* oxidase which contains heme and Cu_B, but lacks Cu_A (e.g. cytochrome *c* oxidase from *Rhizobium*).

In *Shewanella* MR-1 a cluster of four genes was identified which resembles the *ccoNOQP* cluster from *Rodobacter sphaeroides*, *Bradyrhizobium japonicum* and *Vibrio cholerae* which encodes a cbb_3-type cytochrome *c* oxidase.

The CcoN amino acid sequence is highly similar (65-85 % identical) with homologous proteins isolated from the bacteria mentioned above. Based on analysis of known proteins and multiple alignments, CcoN is predicted to be membrane bound, about half the size (296 amino acids compared to 535 in *Rhodobacter sphaeroides*, 549 in *B. japonicum*, 539 in *P. denitrificans*) and highly hydrophobic, containing (by comparison) seven potential transmembrane helices (see Fig 3.10). This subunit is predicted to bind the low spin heme *b*, the high spin heme b_3 and the Cu_B. From 12 histidines, 9 are conserved and only 5 are within the transmembrane helices and will probably bind the hemes and copper ion. The *ccoN* gene in *R. sphaeroides* is regulated by FnrL in response to oxygen concentration (Mouncey & Kaplan, 1998).

```
ccoN_P.denitrif       FIVTIAMLHIVNNLAIPVSLFGSKSVQLFSGVQDAMTQWWYGHNAVGFFLTAGFLGMMYY 288
ccoN_MR1              --------------MAVPVSLF--KSYSMYSGAVDAMVQWWYGHNAVGFLLTAGFLGMMYY 45
fixN_B.japonicum      FIVTIAVLHLGNNPALPVSAFGSKSYVAWGGIQDAMFQWWYGHNAVGFFLTAGFLAIMYY 297
                                    *:*** *  **   :.*  *** ***********:******.:***

ccoN_P.denitrif       FIPKQAERPVYSYKLSIIHFWALIFLYIWAGPHHLHYTALPDWASTLGMVFSIILWMPSW 348
ccoN_MR1              FVPKQAGRPVYSYRLSIVHFWALIALYIWAGPHHLHYTALPDWTQSLGMVMSLILFAPSW 105
fixN_B.japonicum      FIPKRAERPIYSYRLSIIHFWALIFLYIWAGPHHLHYTALPDWTQTLGMTFSIMLWMPSW 357
                      *:**:* **:***:***:****** ******************:.:***.:*::*: ***

ccoN_P.denitrif       GGMINGLMTLSGAWDKLRTDPIIRMMVVAVGFYGMATFEGPMMSIKAVNFVSHYTDWTIG 408
ccoN_MR1              GGMINGIMTLSGAWHKLRTDPVLRFLVVSLSFYGMSTFEGPMMAIKTVNALSHYTDWTVG 165
fixN_B.japonicum      GGMINGLMTLSGAWDKLRTDPVLRMLVVSVAFYGMSTFEGPMMSIKVVNSLSHYTDWTIG 417
                      ******:*******.******::*::**::.****:*******:**.** :*******:*

ccoN_P.denitrif       HVHSGALGWNGMITFGALYYLVPRLWGRERLYSTGLVSWHFWLATIGLVLYAASMWVSGI 468
ccoN_MR1              HVHSGALGWVAMVSIGSLYHLIPVLFGHGRMYSIKLVNVHFWLATIGTVLYIVSMWISGV 225
fixN_B.japonicum      HVHSGALGWVGFVSFGALYCLVPWAWNRKGLYSLKLVNWHFWVATLGIVLYISAMWVSGI 477
                      ********* .::::*:** *:*  :.:  :**  **. ***:**:* ***  :**:**:

ccoN_P.denitrif       MEGLMWREVDAQGFLVNAFADTVAAKFPMNVVRALGGVLYLGGALIMCYNLWATVAK-QP 527
ccoN_MR1              MQGLMWRAVNADGTLTYSFVESLEASYPFYFVRFLGGCFFLTGMLIMAYNVIRTVKA-SK 284
fixN_B.japonicum      LQGLMWRAYTSLGFLEYSFIETVEAMHPFYIIRAAGGGLFLIGALIMAYNLWMTVRVGEA 537
                      ::*****   : * *  :* ::: * .*: .:*  ** ::* * ***.**:  **   .
```

Fig 3.10 Amino acid sequence alignments for the CcoN subunit (the putative transmembrane helices are underlined)

The CcoO protein from *Shewanella* MR-1 is a *c*-type cytochrome with, by analogy with CcoO proteins isolated from other bacteria, a putative transmembrane helix near the N-terminal end (see Fig 3.11) and a *c*-type heme binding motif, totally conserved in the family: C_{66}-Y_{67}-N_{68}-C_{69}-H_{70}-//-M_{138}-P_{139}.

```
fixO_B.japonicum     MSFWTRHQVFEKNSIILIVGILLVIAIGGLVEITPLFYLKSTIEKVDGVRPYTPLELAGR 60
ccoO_P.denitrif      MAILEKHKVLEKNATLLLVFSFLVVTIGGIVEIAPLFYLQNTIEKVQGMRPYTPLELKGR 60
ccoO_MR1             MKFN--HEIVEKNIGLLAIFTVIAISFGSLVEITPLIFQKDTTEPVEGLKPYTALQLEGR 58
                     * :   *::.***  :* :  .:.:::*.:***:**:: :.* * *:*::***.*:* **

fixO_B.japonicum     NVYVREGCYLCHSQMIRPLRDEVERYGHFSLAAESMFDHPFQWGSKRTGPDLARVGAKYS 120
ccoO_P.denitrif      DIYVREGCYVCHSQMIRPMRDEVERYGHYSLAAESMYDHPFQWGSKRTGPDLARVGGRYS 120
ccoO_MR1             DIYVREGCYNCHSQMIRPLRAETERYGHYSVAGESVWDHPFQWGSKRTGPDLARVGGRYS 118
                     ::******* ********:* *.*****:*:*.**::*******************.:**

fixO_B.japonicum     DDWHVTHLTNPRAIVPQSVMPGYPFLSATEVDPDTIADHMRTLRTVGVPYTDDQIANASA 180
ccoO_P.denitrif      DEWHLDHLVDPQAVVPESIMPKYGFLLNRQVDASNMQQRLKTDALGGVPYDDAMIAAAGE 180
ccoO_MR1             DKWHEVHLIDPRAVVPQSNMPAFPWLAENKLDGKLTGDKMTILR--------NMHKGGYK 170
                     *.**  ** :*:*:**:* ** : :*   ::* .   :::                   .

fixO_B.japonicum     DLKAQADPDNAGADAFNKRYAKAVVRNFDGKTGTPTEMDALIAYLQMLGTLVDFKIYNEK 240
ccoO_P.denitrif      DFRVQAAPD-ADASGLEERYPGAQQRNFDRRPG-VSEMDALIAYLQVLGTMVDFSTFEPD 238
ccoO_MR1             GNDLYTDEEIAGAQKAVEG---------------KTEMEALIAYLQSLGHALK------- 208
                     .   :  : *.*.   :                :**:******* **  :.
```

Fig 3.11 Amino acid sequence alignment of CcoO subunits (the heme binding site and conserved methionine are in red; the putative transmembrane helix is underlined)

```
ccoP_MR1             MSSFWSIWISVLSLIVIAGCFLLLRVCSKNTTDVKEGESMGHSFDGIEELNNPLPKWWSY 60
ccoP_V.cholerae      MTTFWSLWIIVITVGTLLGCAILLVWCLKDKMGVEEGVDMGHEYDGIRELNNPLPKWWTY 60
ccoP_P.aerug         MTTFWSLYITALTLGTLLALTWLIFATRKGQRSSTTDETVGHSYDGIEEYDNPLPKWWFM 60
                     *::***::* .::: .: .    *:      *.  .    .  :**.:***.* :*******

ccoP_MR1             MFYITIVFGLVYLALFPGLGNYKGLLNWTSSNQSIGTGQGIKADSAAAVELAAKEGQYVQ 120
ccoP_V.cholerae      LFIGTFIFAAIYLTLYPGLGSFKGILGWQSSDQTVRSLE----ESRASIAAAQQNKQLVQ 116
ccoP_P.aerug         LFVGTLVFAVGYLALYPGLGTWKGLMPGYQSADEFADKE-------------KGWTGVHQ 107
                     :*  *::*.  **:*:****.:**::    .* : .   :                    *

ccoP_MR1             YDQEVKHANEKYGPIFA--AYLATP-----LEELVKNQEALKVGGRLFLQNCAQCHGSDA 173
ccoP_V.cholerae      YSKELDDAEAYYGEAFKRLAYQDGTTNLREIPDIAADSDALKVGQRLFLQNCSQCHGSDA 176
ccoP_P.aerug         WEKEMAKADEKYGPIFA--KFAAMP-----IEEVAKDPQAVKMGGRLFASNCSICHGSDA 160
                     :.:*: .*:  **  *     :    .      : ::. : :*:*:* *** .**: ******

ccoP_MR1             RGSKGFPNLTDSDWLYGGDLATIKTTIMGGRHGMMPPKGGLPIDDSEIAGLAEYVVK-LS 232
ccoP_V.cholerae      RGQKGFPNLTDDAWLYGGEPQAIVTTIRHGRIGQMPAWKDI-LGEQGVKEVVSYTLS-LS 234
ccoP_P.aerug         KGAYGFPNLTDADWRWGGEPETIKTTIMAGRHAAMPAWGEV-IGEEGVKNVAAFVLTQMD 219
                     :*  *******  * :**:  :* ***  ** . **.    : :.:. :  :. :.:. :.

ccoP_MR1             GR---EHDEKLAAQGQGSFMKGCFACHGMDAKGNKLMGAPNLTDD-VWVYGGSRGVIEET 288
ccoP_V.cholerae      GR---SVNAKEAEAGKARFAV-CSACHGTDGKGNPAFGAPNLTDN-DWLFGDSRAEVTET 289
ccoP_P.aerug         GRKLPEGAKADIEAGKQVFATTCVACHGPEGKGTPAMGAPDLTHPGAFIYGSSFAQLQQT 279
                     **    .           *:  *   * **** :.**.  :***:**.    :::*.* . : :*

ccoP_MR1             IKHGRTGVMPAWKDVLGEEKVHVIAAYVYSLSNK----- 322
ccoP_V.cholerae      VMNGRSGVMPAWINTLGEEKIQLVAAYVWSLSNSENK-- 326
ccoP_P.aerug         IRYGRQGVMPAQQEHLGNDKVHLLAAYVYSLSHGEKSAE 318
                     :  ** *****  : **::*::::****:***:
```

Fig 3.12 Alignment of CcoP amino acid sequences from *Shewanella* MR-1, *V. cholerae* and *P. aeruginosa* (the heme binding sites and conserved methionines are in red)

CcoQ is a small membrane protein with a hydrophobic amino acid stretch at the N-terminal end and unknown role (Toledo-Cuevas *et al.*, 1998).

CcoP is a membrane bound *c*-type cytochrome which exhibits lower homology with known proteins (38-50 %), compared with CcoN and CcoO. Two heme *c* binding motifs are found: C_{165}-A_{166}-Q_{167}-C_{168}-H_{169}-//-M_{208}-P_{209} and C_{252}-F_{253}-A_{254}-C_{255}-H_{256}-//-M_{297}-P_{298} (see Fig 3.12).

3.12 Cytochrome *c* peroxidase (ccp111)

Cytochrome *c* peroxidase catalyses the two electron reduction of hydrogen peroxide to water. The protein is 48 % identical to the cytochrome *c* peroxidase isolated from *P. aeruginosa* (Samyn *et al.,* 1995). Based on similarity with cytochrome *c* peroxidase from *P. aeruginosa* and *V. cholerae*, the *Shewanella* MR-1 homologue is a soluble protein (see Table 3.2) which contains two c_4-type hemes, located in two related domains and probably resulting from gene duplication (Hu *et al.*, 1998). The heme located close to the N-terminal end has His_{80} as a sixth ligand and the second heme is coordinated by Met_{279} (see Fig 3.13).

```
ccp_V.cholerae     KLYFDPRLSKSGFISCNSCHNLSMGGSDNLKTSIGHNWQQGPINSPTVLNSSLNIAQFWD 108
ccp_MR1            MLFFEPRLSKSGFISCNSCHNLSTGGVDALPTSIGHHWQEGPINSPTVLNADFMLAQFWD 104
ccp_P.aerug        KLFFDPRLSRSHVLSCNTCHNVGTGGADNVPTSVGHGWQKGPRNSPTVFNAVFNAAQFWD 118
                    *:*:****:* .:***:***:. ** * : **:** **:** *****:*: :  *****

ccp_V.cholerae     GRAADLKEQAGGPIANPGEMAFTHTLAIDVLQSIPAYVSEFRLVFGKP--TLDIDQVTEA 166
ccp_MR1            GRASNLKEQAAGPIANPKEMGFTHELATETIASMPAYRARFAKVYGDE--KVDIDRLTDA 162
ccp_P.aerug        GRAKDLGEQAKGPIQNSVEMHSTPQLVEQTLGSIPEYVDAFRKAFPKAGKPVSFDNMALA 178
                   *** :* *** *** *. **  *  *. :.: *:* *    *  .: .    :.:*.:: *

ccp_V.cholerae     IAEFEKTLVTPYSRFDQWLMGDDSAITAQELAGYELFKNSGCVACHNGSALGGNSFQKMG 226
ccp_MR1            IAAFEKTLVTPNSPFDQYLLGKQDAISGDAKAGYQLFKDKGCVSCHNGPAVGGTMFMKMG 222
ccp_P.aerug        IEAYEATLVTPDSPFDLYLKGDDKALDAQQKKGLKAFMDSGCSACHNGINLGGQAYFPFG 238
                   *  :* ***** * ** :* *.:.*: .:   * : * :.** :****  :**  :  :*

ccp_V.cholerae     LIEPYQTNNKVEGLSA---VTGADADRFKFKVPTLRNVALTYPYFHDGEAATLKDAVDIM 283
ccp_MR1            LIKPFHTNNPAEGRKG---VTGKDADKFVFKVPTLRNIELTYPYFHDGSVWTLEEAVNTM 279
ccp_P.aerug        LVKKPDASVLPSGDKGRFAVTKTQSDEYVFRAAPLRNVALTAPYFHSGQVWELKDAVAIM 298
                   *::  .:.   .* ..   **  ::*.: *:...***: ** ****.*..  *::**  *

ccp_V.cholerae     GRLQLGRKFTDDENGKIVAFLHTLTGEQPSFALPILPPSNDNTPKPQPFD---- 333
ccp_MR1            ADIQLGQKLTEKETKEMVAFLNSLTGEQPQISLPILPPSNKETPRPVPFATGAK 333
ccp_P.aerug        GNAQLGKQLAPDDVENIVAFLHSLSGKQPRVEYPLLPASTETTPRPAE------ 346
                   .  ***:::: .:  ::****::*:*:** .  *:**.*.. **:*
```

Fig 3.13 Amino acid sequence alignment of cytochrome *c* peroxidases from *V. cholerae*, *P. aeruginosa* and *Shewanella* MR1 (the heme binding sites and the conserved sixth ligands are in red)

3.13 Cytochrome *c* reductase (cytochrome bc_1 complex)

The cytochrome bc_1 complex has been isolated from a vast variety of sources: mitochondria and bacteria. The polypeptide composition for the different sources varies from 3 (bacteria) to 11 (mitochondria). Three subunits are conserved throughout all the bc_1 complexes isolated: cytochrome *b*, cytochrome c_1 and a Fe-S protein. All these subunits are highly conserved and can also be found in *Shewanella* MR-1. Cytochrome c_1 contains one heme binding site situated towards the N-terminal end and is 60 % identical to the same protein isolated from *V. cholerae* (see Fig 3.14).

```
c1_V.cholerae       -MKKWIVVLFAMLPSLAMAAGAN-VHLDKANNDLTDQASLQNGAKLFMNYCFGCHSTQYQ 58
c1_MR1              -MKKLLIALVTLLPTLAIAAGGQ-VHLEDXNVDLHDKASLERGLGLFQHYCSGCHSTQYQ 58
c1_P.aerug          MKKQFAALILAVLPVLGFAAGGHGPQLDHVDIDLTDKAAMQDGARTFANYCMGCHSAKFQ 60
                      *:     :.::** *.:***.:  :*:. : ** *:*::: *    * :** ****:::*

c1_V.cholerae       RYERVANDLGIPADLMKENLIFDPETKIGQLMENAIPDKSAAKWFGAPPPDLTLVARVRG 118
c1_MR1              RYERVANDLGISADDMRNKYMFT-DAKIGELMQNAIPPKDAAKWFGATPPDLTLVARVRG 117
c1_P.aerug          RYERVATDLGIPADLMMEKLVFT-GAKIGDHMDIGMKPADAKTWFGAAPPDLTLVARVRG 119
                    ******.****.** * :: :*   :***: *: .:    .* .****.************

c1_V.cholerae       TDWLYTYLRSFYTDPSRPFGVNNIVFPSVGMPHVLEELQGTPEPIFETKVVDGNEVQHVV 178
c1_MR1              EDWVYSYLKGFYKDPSRPFGVNNTVFPSVGMPHVLEELQGTP-------------VKQED 164
c1_P.aerug          TDWLYSYLRSFYEDPKRPWGVNNVIFPNVGMPNVLAPLQGRQVIGCKQVQVVEDGKKQFD 179
                     **:*:**:.** **.**:**** :**.****:**  ***                 ::

c1_V.cholerae       ---G-----------VKSRGNGELSEGEYNQAVRDLVNFLEYSGEPMKLERQNLGWWTMG 224
c1_MR1              ---G-----------TIVVSGGKLNAEEYDQAVRDITGFLVYSAEPVKLERQALGWWVLG 210
c1_P.aerug          PLTGTPLTHEACDQLTVVPKTGELNEAQFDEKVKNLVTFLAYSANPNKLASERIGTYVLL 239
                       *           .     *:*.  :::: *:::. ** **.:* **  : :* :.:

c1_V.cholerae       FLVIFTIVVVALKKEYWRDVH 245
c1_MR1              FLFIFFIVAYLLKKEYWKDVH 231
c1_P.aerug          YLAFFFVFAYLLKREYWKDVH 260
                    :* :* :..  **:***:***
```

Fig 3.14 Amino acid sequence alignment of cytochromes c_1 from *V. cholerae, P. aeruginosa* and *Shewanella* MR-1 (the heme binding site and the conserved methionine are in red; the putative transmembrane helix is underlined)

In the same region, clustered together, probably in an operon, are the genes encoding for cytochrome *b* and a Fe-S centre. In most bacteria, cytochrome *b* is an integral membrane protein with eight transmembrane helices, and contains two different *b*-type hemes (low and high redox potentials) which form an electrical wire spanning the membrane (Montoya *et al.,* 1999). Cytochrome c_1 is anchored to the membrane by a C-terminal transmembrane α-helix (Pereira *et al.,* 1999). The Fe-S centre is associated with the complex via a membrane spanning N-terminal domain (Montoya *et al.*, 1999).

3.14 Oxygen binding cytochrome (Shp208)

In *R. sphaeroides* a protein was identified that transiently binds oxygen during slow autoxidation (Klarskov *et al.,* 1998). The protein has unknown physiological

significance, is not homologous to any gas-binding heme protein, is not abundant in phototrophically grown cells and is not induced by aerobic growth (Klarskov *et al.*, 1998). From the crystal structure it was revealed that an asparagine (Asn_{88}) provides the sixth ligand and that Shp could reduce small ligands like peroxide or hydroxylamine (Leys *et al.*, 2000). Similar proteins have been isolated from *Chromatium vinosum* (Gray *et al.*, 1983) and *Methylophilus methylotrophus* (Klarskov *et al.*, 1999). In *Shewanella* MR-1 an Shp homologue was identified (Shp208) which is 45 and 33 % identical to the Shp proteins from *R. sphaeroides* and *M. methylotrophus* respectively. The protein is soluble, with a typical signal sequence (see Table 3.2) and one heme binding site for which Asn_{120} would probably be the sixth ligand (see Fig 3.15).

```
shp_R.sphaer      -----------GDTSPAQLIAGYEAAAG------APADAERGRALFLSTQTGGK------- 37
shp208_MR1        MNHHLNKHRLELAITAVLLASFNAFAANAAVTSLPLSAERIGLQLQQYQAQGAGPFSADA 60
shp_M.methyl      MKIKTIIAVFGVLFSAHALADVTNAEKLVYKYTNIAHSAN-PMYEAPSITDGKIFFN--- 56
                                .*  :*.                : .         : *

shp_R.sphaer      ---------PDTPSCTTCHGADVTRAGQTR-TGKEIAPLAPSATPDRFTDSARVEKWLGRN 88
shp208_MR1        GQALWLQQSEDRSCTSCHTAKATAQGIHQNTQKPIEAMAPSMTNNRLTDAAKIEKWFSRN 120
shp_M.methyl      -RKFKTPSGKEAACASCHTNNPANVGKNIVTGKEIPPLAPRVNTKRFTDIDKVEDEFTKH 115
                           .  :*::**  . :  *    * * * .:**  . .*:**  ::*. : ::

shp_R.sphaer      CNSVIGRDCTPGEKADLLAWLAAQ----- 112
shp208_MR1        CNWTFKRECTPQEKGDALLWLSLQ----- 144
shp_M.methyl      CNDILGADCSPSEKANFIAYLLTETKPTK 144
                  **  :  :*:* **.: : :*  :
```

Fig 3.15 Amino acid sequence alignment of Shp208(MR-1)and Shp proteins from *R. sphaeroides* and *M. methylotrophus* (the heme binding site and the asparagine are shown in red)

3.15 *c*-type cytochromes with no homologues

Some of the identified cytochromes were not similar to any of the proteins listed in the general protein databases. The heme binding sites and the signal sequences were identified for all of them, but no functional predictions were carried out.

Cyc202 -soluble heptaheme cytochrome with no homology, except with some multiheme cytochromes, at the level of the heme binding sites (see Chapters IV and V)

Cyc260a -soluble protein (see Table 3.2) with 8 heme binding sites. The sites are separated by 18-47 amino acids, except the eighth binding site which is situated close to the C-terminal end and is 173 amino acids away from the previous site.

Cyc260b -soluble protein (see Table 3.2) with one heme binding site situated towards the N-terminal end. Class I cytochrome: 1 heme / 85 amino acids.

Cyc220b -diheme protein devoid of signal sequence, with two heme binding sites in close proximity (18 amino acids) situated in the middle of the peptide.

Cyc208 -soluble diheme cytochrome (see Table 3.2) with 1 heme / 74 amino acids. One heme binding site is located close to the N-terminus, the other close to the C-terminus.

Cyc32 -membrane bound protein (see Table) with two *c*-type heme binding sites (1 heme / 231amino acids) located in close proximity, in the middle of the protein.

Cyc185 - soluble protein (see Table 3.2) with two heme binding sites (1 heme/ 115 amino acids). Shows 46 % identity with a hypothetical protein from *P. aeruginosa*.

Cyc220a - 311 amino acid protein devoid of signal sequence (see Table 3.2), with four heme binding sites, concentrated in the first half of the polypeptide. The

distance between hemes I and II is 27 amino acids and between hemes III and IV is 13 amino acids, which might indicate a c_3-type tetraheme cytochrome.

Chapter IV cyc129 and cyc202 gene isolation

4.1 Why decaheme 129 (cyc129) and heptaheme 202 (cyc202)?

During anaerobic growth, 60 % of the membrane-bound cytochromes are directed to the outer membrane in *Shewanella* MR-1 (Myers & Myers, 1992). As *Shewanella* is able to metabolise Fe(III) and Mn(IV) oxides, it is speculated that this location is important in order for the bacterium to make direct contact with these insoluble substrates. All of the outer membrane cytochromes studied so far in *Shewanella* MR-1 are decahemes involved in Fe(III) and Mn(IV) reduction (see Chapter 3.3). Analysis of the genomic DNA sequence revealed the presence of at least eight membrane-bound decaheme *c*-type cytochromes. Six decahemes are organised in a cluster and the other two, Cyc18 and Cyc129, are membrane proteins which exhibit weak similarities with other decahemes isolated from *Shewanella* MR-1. Analysis of the genome sequence in the regions surrounding the genes does not give any clues as to their possible functions in the cell.

Cyc129 was chosen for further investigation. Database searches showed that the closest homologue is MtrF, another decaheme cytochrome from *Shewanella* MR-1 (see Chapter 3.3). MtrF is part of the *mtrDEF-omcA-mtrCAB* gene cluster. It was demonstrated that the *mtrCAB* operon is involved in Fe(III) and Mn(IV) reduction (Beliaev *et al.*, 2001). It is speculated that *mtrDE*F encodes a second, lower activity, ferric reductase (Beliaev *et al.*, 2001). However, the similarity between the two sets of proteins is low (24 % identity).

Analysis of the amino acid sequence of Cyc129 reveals the presence of a lipoprotein signal sequence (see Table 3.4 in Chapter III) which will direct the protein to the membrane (probably the outer membrane). The mature polypeptide has an estimated

molecular weight of ~79 kDa, an isoelectric point of 5.7 (as calculated by the ProtParam Tools programme) and consists of 737 amino acids. Cyc129 is probably organised into two domains, each containing 5 hemes situated at equivalent distances, as shown in Fig. 4.1.

211 aa - **H1** - 21 aa - **H2** - 55 aa - **H3** - 14 aa - **H4** - 23 aa - **H5**

195 aa - **H6** - 18 aa - **H7** - 69 aa - **H8** - 33 aa - **H9** - 32 aa - **H10** - 16 aa

Fig 4.1 Organisation of Cyc129. H= heme binding site; aa= amino acids

There are only 5 methionine residues in the mature protein, one of which is involved in the CxxCH heme binding motif. Thus the sixth ligand for each of the hemes is most probably a histidine residue. From the 28 histidine residues present, 10 are involved in the CxxCH motif. From the amino acid alignment between MtrF and Cyc129, 10 histidines are conserved: 243, 287, 290, 353, 381, 581, 632, 635, 715 and 754 (see Fig 4.2) and these could provide the sixth ligands for each of the ten hemes.

```
129_MR1         MMKNYN--KSLLALALTSALCLTACGDGEDGKDGAPGTPGTPGTPGTPGLPAGSFAKTAE 58
MtrF_MR1        -MNKFASFTTQYSLMLLIATLLSACG-GSDGDDGSPGEPGKP----------------PAM 43
                 *:::   .:  :* *  *  *:*** *.**.**:** **.*                .*

129_MR1         SITDLKFTLSPADIKVTSSEGFSVKFTLTGKSSGKDVPFMGLDKIAVYSLTANENTSGTG 118
MtrF_MR1        TISSLNISVD----KVAISDGIAQVDYQVSNQENQAVVGIPSATFIAAQLLP-QGATGAG 98
                :*:.*::::.    **: *:*::      ..:...: *  :   .: . .* . :.::*:*

129_MR1         APIEWQNNATANKAGSSLTCTLNGLNGTKNACTLKEDAANPGTYTATWTYDGAAPIINPN 178
MtrF_MR1        NSSEWQHFTSETCAASCPGTFVDHKNGHY-------------SYRFSATFNGMNGVTFLS 145
                 . ***: :: . *.*.    ::  **                :*  : *::*   :   .

129_MR1         DNPNAVHRVFVRAYNIVNSQGVALADKVLSVPVDFIPTTDELAASGKDTVSSAACKNCHG 238
MtrF_MR1        D---ATQRLVIKIGGDALADGTVLP--ITNQHYDWQSSGNMLAYT-RNLVSIDTCNSCHS 199
                *   *.:*:.::  . . ::*..*.  : .   *: .: : ** : :: **  :*:.**.

129_MR1         EVDGHIAKIEAHHNYQDVKNCVTCHNPDLVPSDAQLAEGWVFDFAPMIHRIHAGEHNAAY 298
MtrF_MR1        NLAFHGG------RYNQVETCVTCHNSKKVSNAADIFP-------QMIHSKHL------- 239
                ::  * .      .*::*:.******.. *.. *::         ***  *

129_MR1         LSGEAKEYFGEIGFPSDLKECKSCHDGAPSYNTN------IYAQACVGCHINVNFATGEN 352
MtrF_MR1        -----------TGFPQSISNCQTCHADKPDLADRQNWYRVPTMEACGACHTQINFPAGQG 288
                           ***..:.:*::** . *.    .         :** .** ::**.:*:.

129_MR1         HSEFGLAQADDTQCKSCHGSGSLTPEAVHSVGKRAEYADLFKVDFTSAAVVPSATLGMKT 412
MtrF_MR1        H----PAQTDNSNCVACHN--ADWTANVHS-------------NAAQTSALAQFNASISS 329
                *    **:*:::* :**.  :       **             : :.::.:.. . .:.:

129_MR1         LTLKANVSINGAPIADGTSLATYHATNNPTGKLVANGLLLGNVATDGTVYAWRDVKPTAI 472
MtrF_MR1        ASMDANGTITVAVSLTNPTTGTAYADSADKLKFISD---LRIYANWGTSFDYSSRSARSI 386
                 ::.** :*. *    ..: .* :* .  . *::::   *   *. ** : : . .. :*

129_MR1         SLNLASGTLSGGVLTFVKDIPDAQAIGTIYVSSEANACIKAGAVTSCNATGLEFGPTNPI 532
MtrF_MR1        RLPEST-PIAGSNGTYSYNISGLTVPAGTESDRGGLAIQGRVCAKDSVLVDCSTELAEVL 445
                 *  :: .::*.  *:  :*.. . .    . . *     .....  .. .    :: :

129_MR1         GN SSPVKFFSLDGSAVSTARMADPSRITVEEAKCNACHGTLDYIKGTRHGTYTF T QC 589
MtrF_MR1        VIKSSHSYFNMS-------ALTTTGRREVISNAKCASCHG--D-QQLNIHGARNDLAGQC 495
                   **   *            *  :   * .:. *** :***  *     . **: .  : **

129_MR1         MNCHNDTTGASGHKTVVYKGDDGSKVVNPDVTFNNKDLFTVAHRFHSGNFDSITGIFRNA 652
MtrF_MR1        QLCHN--------------PNMLADATATNPSMTSFDFKQLIHGLHSSQFAGFEDLN--- 538
                  ***               :  :... .  : .. *   : *  **.:* .:  *

129 MR1         LEGYPSPETACSACHK-DSAKLFATDGGLTSEKRSIKVG-SNYISPVAESCRSCHAHSDA 710
MtrF_MR1        ---YPGNIGNCAQCHINDSTGISTVALPLNAAVQPLALNNGTFTSPIAAVCSNCHSS--D 593
                   **.     *: **  **: : :.   *.:  :.: :. ..: **:*  * .**:

129_MR1         AAVAHFRSNGAIVEADAVTDSNLPVESCATCHAEGKTYGIDKVHAEVAH 759
MtrF_MR1        ATQNHMRQQGAVFAG-TKADATAGTETCAFCHGQGTVADVLKVHPIN-- 639
                *:   * *. :**:.               *.*.:** .:*.      : ** *.
```

Fig 4.2 Amino acid sequence alignment of MtrF and Cyc129 from *Shewanella* MR-1

(the heme binding sites and the sixth ligand are shown in red)

Cyc202 is the only heptaheme cytochrome identified in *Shewanella* MR-1 and is a soluble protein, as indicated by the signal sequence and the lack of internal hydrophobic sequences (see Table 3.2). The mature protein contains 677 amino acids and has an estimated molecular weight of ~79.2 kDa and an isolectric point of 6.5. There are 18 histidine residues that are not part of the CxxCH motif and 19 methionine residues, any of which could function as a sixth ligand of the heme iron.

There is only one report of a protein containing seven heme binding sites, ORF3, isolated from *Methylococcus capsulatus* (Bergmann *et al.*, 1999), but the two sequences are only weakly related (see Fig 4.3). ORF3 from *M. capsulatus* is part of a gene cluster composed of ORF1 (nonaheme cytochrome), Occ (octaheme c_{5530}), ORF3 (heptaheme) and ORF4. The role of these proteins is unknown. Occ may be induced by ammonia and two promoter sequences upstream c_{5530} are similar to NtrA-dependent promoters, which might indicate a role in nitrogen metabolism (Bergmann *et al.*, 1999).

Upstream of *cyc202* gene there are numerous genes encoding proteins involved in nitrogen metabolism but whether this cytochrome is involved or not it is not known.

```
cyc202_MR1          --------MMKRWKTKTALGVLFCLGSAVSATTIASDAKSDGKVVPGVGNKQQTHYTQDI 52
M.capsulatus        MWIHRLQICPWLWAVCFIAGILPSYGGEAPADNGFDRAVLHP-AIPLLDESGRHVLDSGL 59
                                * .    *:* . *. ..* .  . *  .  .:* :.:. :    ..:

cyc202_MR1          LANPKVSEN---------LMEKSRGVKTLQDYIVQEQELFDFL----------FENHPVF 93
M.capsulatus        PYSPKNSCGNGSGSGCHDYARITRGYHFEQGRDETRDGFGNKLGLPQLTGPGYFGGYNCM 119
                      .** * .           . :** :  *.    .: : : *            * .:  :

cyc202_MR1          KYDAEGRLKGTYKVSDRGEEYLHGGDSVAYS---------------------KHSKEVNS 132
M.capsulatus        SGNAPGWLARKSNGSAAEFGDFGAPDLVRYCGACHSGGGWGEFDRNGGRYDEQSAETVKA 179
                    . :* * *  . : *       : . * * *.                    : :: *::

cyc202_MR1          TDGTAVRYSAYEDGQRPKALQYRLGAKSILDFPNKFVGPEKCGECHG------------- 179
M.capsulatus        FDGDYFSRQFQEPGKTGQYGGSGPSEVVAWDWRRSGVREADCMLCHADFSRLKIFPPSGL 239
                     **  .  . * *:  :       .     *: .. *   .*  **.

cyc202_MR1          ---------PQYEKWR----------RSRHSKTIRFPGEHPEVDN--------------- 205
M.capsulatus        GTGGSESAALQFARLRDEKFIAGGFFRHAASAIWEFLDVRPDTEGGAALLAVERTPATGT 299
                              *: : *           *   *   .* . :*:.:.

cyc202_MR1          ----------DLKKPMYTTKDTSILPSGITPDAIYATVGTPRTKYGFIDAYLVRGTY--H 253
M.capsulatus        ATPDYRLVLDDQGNPKLHWNRDAFDESGKIQVPMLRFPASDNCMYCHKTGNSRRGFYGFG 359
                              *  :*    :  ::  **    .:    .: .  *  .  .    ** *

cyc202_MR1          VKDGLLKDGTGTMVAGGNQFSRGWAEWLTPEMAAKIN------KAIPSFPLKMEDFGTSG 307
M.capsulatus        PEVRVRMAGDGTTITDFRTDVHKGAVWTEDNGQARVIDNCNACHARQYYKSPAANVDLDA 419
                     :  :   * ** ::. .   :  * *   :  *::       :*   :     :.. ..

cyc202_MR1          SHQWGMSSYGAKYEKEFLFQPASSYCEMCHS----------FKFDFQTKEEFFAALGNPK 357
M.capsulatus        DHNFPKGNGDNDVRNDLDNAPPPASCEHCHDQAAKPALPSGHKNVLEAHREIWKANGDMR 479
                    .*::  .. . . .:::    *..: ** **.          .*  ::::.*:: * *: :

cyc202_MR1          ELQKHTISKG-------ITCEECHGAGGHLDG-----------GIGG------------- 386
M.capsulatus        GYPENTLDRITQTHLNVVACQTCHISRLADNGKEFPMRYRYRVGYGGRLKIFPYKPAYRY 539
                       ::*:.:          ::*: ** :     :*          * **

cyc202_MR1          ----GMPSNCERCHQRFNFVEELAETPQGQE--KLEYAFNVKMKSSCPSCGTEGS----- 435
M.capsulatus        FVQDRTSGRVLNRYERFSVIEERTGSDGGNYGAILEPASGKELGRVVMNGDEFGEPPTFA 599
                          ...  . ::**..:** : :  *:    ** * . ::      . .  *.

cyc202_MR1          ------QMFASAHYDKGMRCSTCHDPHEVTDGDWKSGTTKP-KTTKECTDCHTAQAETAK 488
M.capsulatus        DYKALKQAYDALLGMKGYAMPNVRFVYIESNEYALSHATRPSPQAVQCEDCHARKQSGAF 659
                          * : :    **    .. :  :  ::     *  *:*     :* ***: : . *

cyc202_MR1          NTNTHSN------QTCQSCHMPNMGSCEN------FTAIQFPDMAGFDNVRKSHMWKIDV 536
M.capsulatus        SALISAEGLLGEANVAEVAKLPDRRLVDAGIVELGMPYYKVQDDGRIVENVADVLYASRL 719
                    .:  ::      :..: .::*:    :          :.  :. * . : :   . ::   :

cyc202_MR1          DPLRKTLNPPEGKSRD-----------------ATTKGWTVAKDENGYNYLDLMWTCART 579
M.capsulatus        DPSMSILRSETARTVENEFKTLSRAEALAFADLDEAAGQKLAADLPSGEALLFGSKVGHS 779
                    **  . *..  .:: :                 : * .:* *  . : * :  . .::

cyc202_MR1          SASDHDVTENKGCHSQFQSELEVG------LHFEDQMEIYGE------VQKWQKPVKDLF 627
M.capsulatus        SLRGFALIQTRGTRTLAYGDVLKGRVESRPAKAKDRTRIFGQGFGNLVADIYSLAVMDAS 839
                    *  .. : :.:* ::   .::  *         : :*: .*:*:       .: :. .* *

cyc202_MR1          GQVLQGLQRIDKLLEVTQLPVDKKTEVLMLTDKAQDVIKLVEADGSWGAHGPRYTQKRLD 687
M.capsulatus        GRTLPGLVEGTALVRLPYRGKAKARGGVNVLVSNDGKVWQRVGGKNLLVFRPRGDVD--G 897
                    *:.* ** .   *:.:.      *     : :  . :. :     .. .  .. **   .  .

cyc202_MR1          AALTYVQQAQAIIDGNGYNAKM- 709
M.capsulatus        YVVVRIRRSALYLDPRRQGGLKP 920
                     .:. ::::   :* .  ..
```

Fig 4.3 Amino acid sequence alignment of Cyc202 from *Shewanella* MR-1 and ORF3 from *M. capsulatus* (heme binding sites are shown in red)

4.2 Gene isolation and cloning into the pGEM-T vector

Using *S. putrefaciens* MR-1 genomic DNA as a template (see Chapter 2.8.1) the *cyc129* and *cyc202* genes were PCR amplified using two different sets of primers: 129L/129R and 202L/202R (see Materials and Methods). These primers introduced *Bam*HI/*Eco*RI and *Bam*HI/*Sma*I restriction sites respectively, at the ends of the fragments. The PCR reaction was performed using 5 pmol of each primer and 1.25 U of *Taq* polymerase (Promega) using the following parameters: 1 min at 95 °C, 30 cycles of 30 sec at 95 °C, 30 sec at 40 °C, 45 °C at 72 °C and a final extension step at 72 °C for 5 min. After amplification, each reaction mix was checked by electrophoresis on a 0.8 % agarose gel. Fragments of the expected size (2.3 and 2.1 kb respectively) were identified. Taking advantage of the non-template dependent addition of a single deoxyadenosine at the 3' end of PCR products by *Taq* polymerase, the pGEM-T vector (Promega) was used for cloning. This vector derives from the pGEM-5Zf(+) vector which was linearized at the *Eco*RV site and one deoxythymine was artificially added at the 5' end. This modification allows the direct cloning of PCR products, in the presence of 1U T4 DNA ligase (Promega), without the need for restriction digestion of either vector or insert. After ligation for two hours at room temperature, the mixture was transformed into competent *E. coli* TG1 cells which were plated onto LB+Amp+X-Gal+IPTG plates (see Chapter 2.7.5). Following overnight incubation at 37 °C, white colonies were selected for further screening. Plasmid DNA was isolated and digested with either *Bam*HI and *Eco*RI or *Bam*HI and *Sma*I to check the size of the insert. The plasmids created were pDA2 for *cyc129* and pDA3 for *cyc202*.

4.3 Cloning of *cyc129* into pMMB503EH

For protein overexpression in *S. putrefaciens* MR-1, the broad host range vector pMMB503EH (Michel *et al.*, 1995) was used. pDA2 was cut with *Bam*HI and *Pst*I and the required 2.3 kb fragment cloned into the 9.9 kb pMMB503EH cut with the same enzymes. The ligation mixture was transformed into competent *E. coli* DH5α cells and cells selected for streptomycin resistance. Plasmid DNA was isolated and digested with *Bam*HI and *Pst*I restriction enzymes (see Fig 4.4).

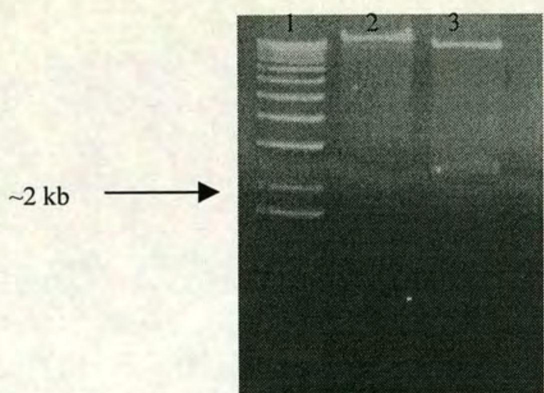

Fig 4.4 Restriction digest of pex129 (*cyc129* in pMMB503EH).
Lane 1- 1 kb DNA marker (Gibco BRL); Lane 2- pex129 digested with *Bam*HI; Lane 3- pex129 digested with *Bam*HI and *Pst*I

4.4 Cloning of *cyc202* into pMMB503EH

For *cyc202*, an attempt was made to clone the gene into pMMB503HE cut with *Bam*HI and *Sma*I, to maintain the correct orientation of the insert relative to the *tac* promoter. For unknown reasons this failed, in spite of numerous attempts. The absence of useful restriction sites required the prior cloning of the 2.1 kb *Bam*HI/*Sma*I *cyc202*

into pBluescript cut with *Bam*HI and *Eco*RV, with the formation of pDA3-5. The *cyc202* gene was excised with *Bam*HI and *Hind*III and cloned into pMMB503EH cut with the same enzymes, to form pex202 (see Fig 4.5).

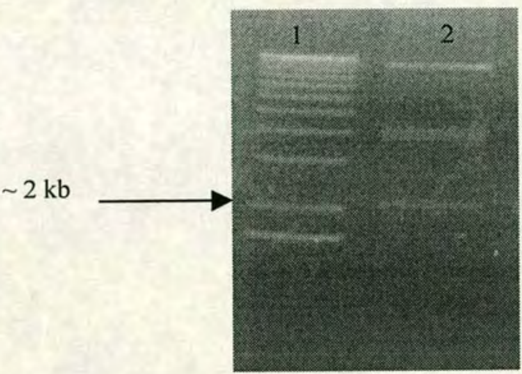

Fig 4.5 Restriction digest of pex202 with *Bam*HI and *Hind*III.
Lane 1- 1 kb DNA marker (Gibco BRL); Lane 2- pex202 digested with *Bam*HI and *Hind*III

4.5 Cyc129 protein overexpression in *Shewanella* MR-1

The pex129 plasmid was transformed into *E. coli* SM10 prior to conjugation with *S. putrefaciens* MR-1, as described in Materials and Methods. To test for expression of the protein, an overnight culture of DA129 (pex129 in *Shewanella* MR-1) was used to inoculate 5 ml Bijoux bottles containing LB and the appropriate antibiotics. At OD_{600nm} ~ 0.8, the cells were induced with 0.01-2 mM IPTG. After 4 hours, the cells were harvested and the pellets resuspended in TE buffer pH 7.5 (see Chapter 2.8.2). Equivalent protein concentrations were loaded on a 10 % SDS-PAGE gel along with a broad-range protein marker (New England BioLabs). After electrophoresis, the gel was

heme stained (see Chapter 2.16). When sufficient colour had developed, the reaction was stopped by rinsing the gel with distilled water. A band of about 80 kDa was identified in the induced samples (see Fig 4.6).

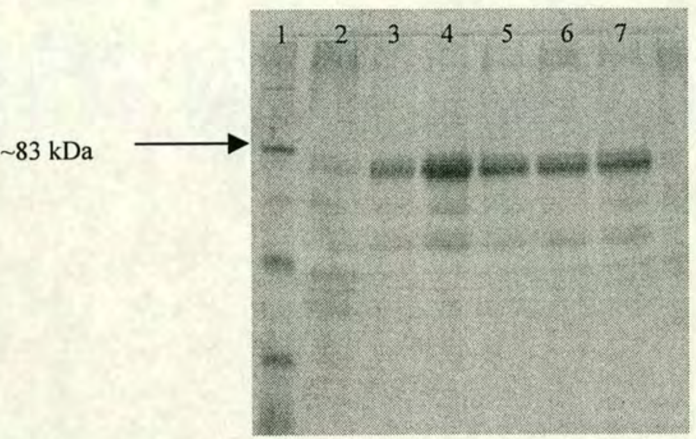

Fig 4.6 10 % SDS-PAGE heme-stained gel of DA129 induced cells.

Lane 1- Broad host range protein marker (NEB); Lane 2- DA129 uninduced cells; Lane 3- DA129 induced with 0.01 mM IPTG; Lane 4- 0.3 mM IPTG; Lane 5- 0.5 mM IPTG; Lane 6- 1 mM IPTG; Lane 7- 2 mM IPTG

The signal sequence indicates a membrane localisation for decaheme 129 (see Table 3.4). To confirm this, the samples were sonicated for 3 x 10 sec and ultracentrifuged (1 hour, 100000rpm), to ensure an efficient separation of the soluble and membrane fractions. The volume of the supernatant was measured and the pellet resuspended in an equal volume of TE buffer pH 7.5. Samples were loaded on a 10 % SDS-PAGE gel and after electrophoresis, the gel was heme stained. The overexpressed protein was found in both the soluble and membrane fraction (see Fig 4.7). It is likely

that the sonication step caused the release of some of the Cyc129 into the soluble fraction, which could indicate that the protein is loosely associated with the membrane.

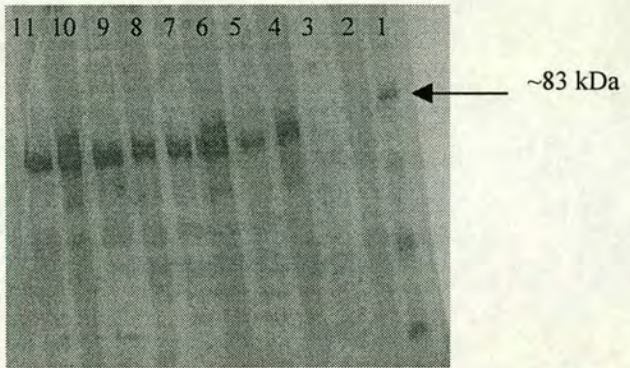

Fig 4.7 10 % heme stained SDS-PAGE gel of sonicated and ultracentrifuged DA129 samples.

Lane 1- Broad range protein marker (NEB); Lanes 2, 4, 6, 8, 10- soluble fractions of uninduced, 0.3, 0.5, 1 and 2 mM IPTG respectively; Lanes 3, 5, 7, 9, 11- membrane fractions of uninduced, 0.3, 0.5, 1 and 2 mM IPTG respectively

4.6 Cyc129 overexpression in *E. coli*

Although the overexpression in *Shewanella* was successful, the purification of a cytochrome would be much easier in an *E. coli* strain, due to the low cytochrome background. As the gene was already cloned into the pMMB503EH vector, the pex129 construct was used. To our knowledge, this vector has not been used for overexpression in *E. coli*. These cells do not support the production of *c*-type hemes in high quantities (Reincke *et al*., 1999). This problem could be overcome if the genes involved in heme bio-synthesis are supplied on a separate replicon such as plasmid pEC86 (Arslan *et al*., 1998) This plasmid contains the *ccm* genes involved in **c**ytochrome ***c*** **m**aturation and

was co-transformed with the pex129 construct into *E. coli* DH5α. An overnight culture was used to inoculate LB medium supplemented with the appropriate antibiotics. At OD_{600nm} ~ 0.6, the cells were induced with 0.1-2 mM IPTG. After four hours the cultures were harvested and the pellets resuspended in TE buffer pH 7.5. Equivalent protein concentrations were loaded on a 10 % SDS-PAGE gel and after electrophoresis the gel was heme stained. The signal was weak and about the same amount of Cyc129 was produced in the induced and uninduced cells. A change in the induction parameters (cell density, induction time, IPTG concentration) and the addition of 4 mM γ amino levulinic acid (precursor in heme biosynthesis) did not improve the expression. There are two obvious explanations for the low levels of expression: either the amount of correctly folded cytochrome was very low or pMMB503EH is not a good expression vector for *E. coli* strains. The overexpression of Cyc129 in *E. coli* could possibly be improved if the gene were to be cloned into a more specialised expression vector like the pET plasmids. In any case, the *E. coli* host must contain the pEC86 plasmid as well, to avoid the formation of the inclusion bodies.

4.7 Purification trials of decaheme 129

Using strain DA129, a preliminary purification trial was performed. The cells were grown with good aeration in two flasks, each containing 500 ml LB medium supplemented with the appropriate antibiotics. At OD_{600nm} ~0.8, the cells were induced with 0.5 mM IPTG. After 4 hours the cells were harvested and the pellet was resuspended in 10 mM Tris buffer pH 8. After sonication and centrifugation, the soluble

fraction was loaded onto a 20 ml DEAE-Sephadex column (BioRad), equilibrated in 10 mM Tris buffer pH 8. The column was then washed with two volumes of 10 mM Tris buffer pH 8 and the proteins eluted with a gradient of 0-500 mM NaCl in 10 mM Tris buffer pH 8. The fractions were checked on a heme stained 10% SDS-PAGE gel (see Fig 4.8). The protein eluted at ~ 200 mM NaCl in 10 mM Tris buffer pH 8

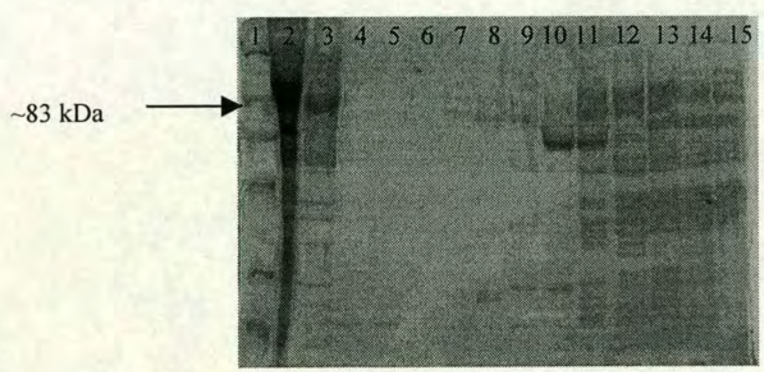

Fig 4.8 10 % heme stained SDS-PAGE gel with samples from DEAE-Sephadex column chromatography

Although a small part of the protein did not bind to the column, due to overloading (lane 3), enough protein was left to be detected on the heme stained gel (lanes 11-13). The decaheme eluted at ~ 200 mM NaCl in 10 mM Tris pH 8. The protein is not pure and further purification steps are required but time constraints forced us to leave the project at this stage.

This preliminary trial demonstrated that although Cyc129 presents a lipoprotein signal sequence (see Table 3.4), during sonication part of the protein is dislocated from the membrane which indicates a loose association.

Chapter V FccA56-isolation and characterisation

5.1 FccA56- a putative acrylate reductase

5.1.1 Organisation of the Fcc56 cluster

Genome analysis revealed the presence of four fumarate reductase-like proteins (see Chapter 3.4) encoded in operons with two (Fcc54 and Fcc342) or three polypeptide sequences (Fcc56 and Fcc63).

The *fcc56* cluster contains three genes that encode for a flavoprotein (FccA56), a tetraheme *c*-type cytochrome (FccB56) and a histidine ammonia lyase-like protein (Hal56), as shown in Figure 5.1.

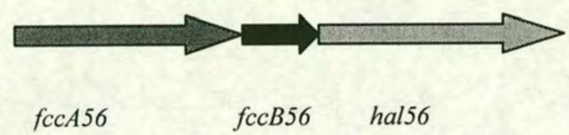

Fig 5.1 Gene organisation of *fcc56* cluster.

FccA56 is 41 % identical to the FrdA flavoprotein subunit of *W. succinogenes* fumarate reductase and 31 % identical to the flavin domain of *S. frigidimarina* NCIMB400 fumarate reductase (see Chapter 3.2). The presence of a double arginine signal sequence (see Table 3.3) usually indicates that the protein is transported to the periplasm in a folded conformation, with the FAD group attached at the active site (Berks 1997). The FccB56 subunit is a tetraheme *c*-type cytochrome and the signal sequence indicates a periplasmic localisation of the mature protein (see Table 3.2). This organisation is not uncommon. In *W. succinogenes*, a flavoprotein (FccA) and two tetraheme cytochromes (FccB and FccC) are located in a cluster. The FccA subunit,

although induced when the bacterium is grown with fumarate and sulfide, does not catalyse fumarate reduction in the presence of viologen radicals (Simon *et al.,* 1998b). *G. sulfurreducens* contains a periplasmically located methacrylate reductase, which requires both subunits (flavoprotein and cytochrome) for catalysis. The two subunits are tightly associated and catalyse the hydrogenation of α, β-unsaturated monocarboxylic acids like methacrylate, acrylate, crotonate, pentenoate, but not the reduction of the cinnamic acid derivatives or fumarate (Mikoulinskaia *et al.*, 1999). In addition to these two subunits, 100 bp downstream is a third reading frame encoding a protein (Hal56), which resembles histidine ammonia lyase from *P. putida* (26 % identical; see Chapter 3.2), the first enzyme in the histidine degradation pathway. This organisation is different from Fcc63, where the Hal subunit is situated between the flavoprotein and the cytochrome.

5.1.2 FccA56 active site

To study this gene cluster, the first protein (FccA56) was isolated and characterised. A molecular modelling study was carried out in order to identify putative residues that form the active site and to postulate a possible function for this protein. Optimised pairwise sequence alignment of FccA56 against Fcc_3, including secondary structure prediction was used for molecular modelling. The structural identity between the two proteins is 74 % (Bilsland, unpublished results). Homology modelling using the Sybil Composer programme was used to predict the three dimensional structure of the

catalytic domain, using the known active site structure of Fcc_3 as a molecular framework (see Fig. 5.2).

```
fccA56            SIRALPVMLDMYQLGPWATPDEKGAGPASFFADYAFAEGIAIDPKTGARFMNELADRRTR 327
fcc3_NCIMB400     NAGGALKDMQYIQAHPTLSVKGGVMVTEAVRG-----NGAILVNREGKRFVNEITTRDKA 405
                                 365         377 381                     402

fccA56            ADAQLAVLASGTKEKPNMPFVFCGEATANHAEGFKAAYRDGAIKKSETLEELAKRYDVDI 387
fcc3_NCIMB400     SAAILAQTG-------KSAYLIFDDSVRKSLSKIDKYIGLGVAPTADSLVKLGKMEGIDG 458

fccA56            NALQNSINEWNEIVQGKAKDPFNKPLDEKTILKPPYYSIRLSPKLHYCMGGVAITPNAEV 447
fcc3_NCIMB400     KALTETVARYNSLVSSGKDTDFERPNLPRALNEGNYYAIEVTPGVHHTMGGVMIDTKAEV 518
                                                               504

fccA56            IDSNTCEPISGLFAAGEVTGGTHGMDRLGGCSSIDGLVFGQIAGNQAAIRKV-- 499
fcc3_NCIMB400     MN-AKKQVIPGLYGAGEVTGGVHGANRLGGNAISDIITFGRLAGEEAAKYSKKN 571

                                       544
```

Fig 5.2 Prediction of the amino acid residues (numbered) involved in the active site, based on homology with Fcc_3 from *S. frigidimarina* NCIMB400

In Fcc_3, the active site is at the interface between the flavin-binding and clamp domains. The C_1 carboxylate group of fumarate is hydrogen-bonded to His_{365} and Thr_{377} and the C_4 carboxylate to His_{504} and Arg_{544} (see Fig 5.3a). The catalytic proton donor is Arg_{402} (Taylor *et al.*, 1999).

Although Fcc_3 and FccA56 are very similar, some of the amino acids involved in fumarate binding and which are well conserved throughout the fumarate reductase family, are not found in FccA56. One end of the binding pocket is completely conserved (His_{504}, Arg_{544}), as is the catalytic arginine residue (Arg_{402}). However the region involved in binding the C_1 carboxylate, involving His_{365} and Thr_{377}, is different (see Fig 5.3b). These residues are replaced by glycine and phenylalanine respectively.

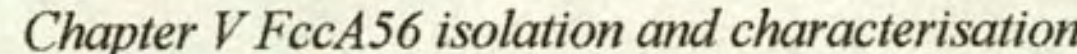

Fig 5.3 The structure of the Fcc_3 (a; Taylor *et al.,* 1999) and FccA56 (b; as predicted by Bilsland) active sites (*S. frigidimarina* NCIMB400 numbering). In Fcc_3 the C1 carboxylate group of fumarate (malate-like substrate in the picture) is twisted out of plane by the closure of the clamp domain and the steric constraints imposed by the side chains of Met_{236} and Met_{375} and by hydrogen bonding to His_{365}. The substrate C4 carboxylate is bound by Arg_{544}, Arg_{402} and His_{504}.

This configuration is perhaps more likely to accommodate monocarboxylate substrates, rather than dicarboxylates, as in the case of Fcc_3.

Based on this secondary structure prediction, it was postulated that FccA56 might reduce substrates like acrylate, methacrylate and their derivatives. These all occur in nature as degradation products of organic matter.

5.2 Isolation of the *fccA56* and *fccB56* genes

The *fccA56* gene was isolated by PCR using *Shewanella* MR-1 genomic DNA as the template. The primers used (A56L and A56R) were designed to amplify a 1.5 kb fragment, including the double arginine signal sequence, with *Bam*HI and *Sma*I restriction sites at the 5' ends. For *fccB56*, primers B56L and B56R were used, which amplified a ~ 0.4 kb fragment, including the signal sequence, which contained *Sma*I and *Bam*HI restriction sites at the 5' end. The cycling conditions were as follows: 1 min at 95 °C, 30 cycles of 30 sec at 95 °C, 30 sec at 40 °C, 45 sec at 72 °C and a final extension step at 72 °C for 5 min. Upon completion, the reactions were checked on a 0.8 % agarose gel and the PCR products cloned into the pGEM-T vector, as described in Materials and Methods and Chapter 4.2.

5.3 Cloning of *fccA56* into pMMB503EH and protein overexpression in *E. coli* DH5α and *Shewanella* MR-1

The same procedure used in Chapter 4.3 was carried out for FccA56 overexpression. The 1.5 kb gene was extracted as a *Bam*HI/*Pst*I fragment and cloned into pMMB503EH cut with the same enzymes, to form pexA56 (see Fig 5.4).

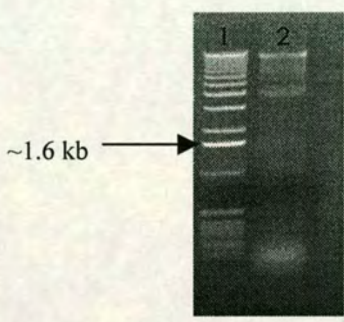

Fig 5.4 Restriction digest of pexA56 with *Bam*HI and *Pst*I enzymes

Lane 1- 1 kb DNA marker (GibcoBRL); Lane 2- pexA56 cut with *Bam*HI and *Pst*I

For expression in *Shewanella* MR-1, pexA56 was transferred into *E. coli* SM10 prior to conjugation (to generate strain DA56). Induction trials were performed in the same manner as for Cyc129: an overnight culture (0.2 ml) was used to inoculate 5 ml Bijoux bottles containing LB medium supplemented with the appropriate antibiotics. When OD_{600nm} was ~ 0.8 (0.5 for *E. coli*), the cells were induced with 0.1-2 mM IPTG. After 4 hours, the cells were harvested and the pellet resuspended in TE buffer pH 7.5. Equivalent protein concentrations were loaded onto a 10 % SDS-PAGE gel. After electrophoresis the gels were stained with Coomassie-Blue and destained in destaining solution (see Materials and Methods).

As shown in Fig 5.5, FccA56 is expressed at high IPTG concentrations (2 mM) in *Shewanella* MR-1. A band corresponding to ~ 50-55 kDa (the calculated molecular weight is ~52 kDa, if the signal sequence is cleaved at the site indicated in Table 3.2) was observed when 2 mM IPTG was used, compared with the uninduced cells.

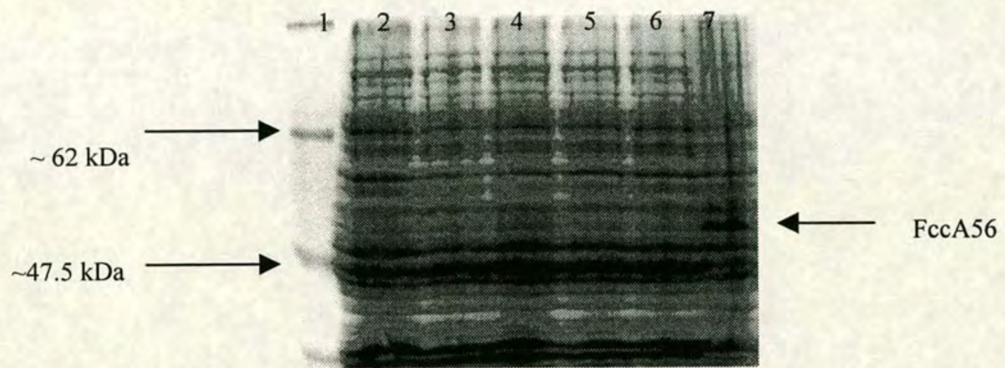

Fig 5.5 Coomassie-Blue stained 10 % SDS-PAGE gel showing expression trials of DA56 (pexA56 in *Shewanella* MR-1)

Lane 1-NEB broad-range marker; Lane 2- DA56 uninduced; Lane 3- 0.1 mM IPTG; Lane 4- 0.3 mM; Lane 5- 0.5 mM; Lane 6- 1 mM; Lane 7- 2 mM IPTG

For purification trials, 5 litre cultures of strain DA56 were grown at 23 °C until OD_{600nm} ~ 0.8. The cells were induced with 2 mM IPTG for 4 hours. After harvesting, the pellets were resuspended in 10 mM phosphate buffer pH 7. As the starting point in the purification, we looked at the estimated isoelectric point which was ~ 8.2 (ProtParam Tools programme). In phosphate buffer pH 7, the pH is less than the pI and the protein should have a net positive charge.

The soluble cell extract was loaded on a 20 ml DEAE-Sephadex column. The collected fractions (breakthrough material) were run on a 10 % SDS-PAGE gel (not shown) and contained only a few proteins, including FccA56. Analysis of the gel indicated that a gel filtration S-200 column might be a useful next step. Unfortunately, chromatography on a S-200 column did not resolve FccA56 from other proteins and improvement in purification was minimal (see Fig. 5.6). Even the use of a longer column (from 60x1 to 120x1 cm) did not improve the separation.

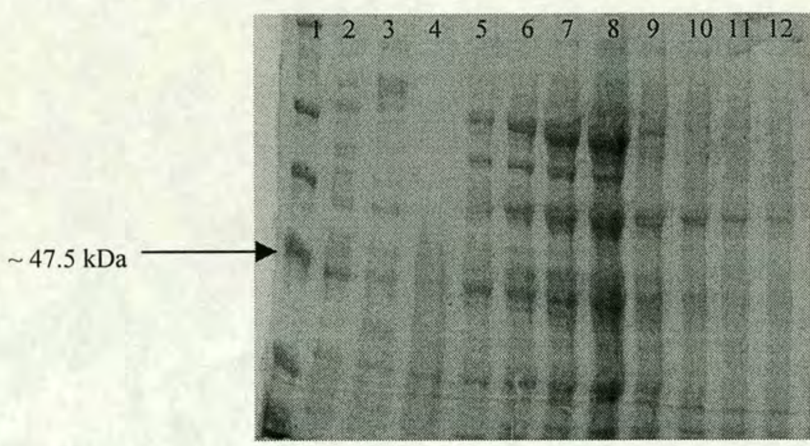

Fig 5.6 Coomassie-Blue stained 10 % SDS-PAGE gel for DA56 purification after gel filtration.

Lane 1- Broad-range protein marker (NEB); Lane 2-12- fractions from gel filtration

Considering the difficulties in the purification of FccA56 overexpressed in *Shewanella* MR-1, a new approach was developed. As the gene was already cloned into an expression vector and pMMB503EH had not been used before for overexpression in *E. coli*, the pexA56 construct was transformed into *E. coli* DH5α competent cells.

Despite numerous trials, involving changing the induction times and the cell density at which the IPTG was added no expression was observed as evidenced by Coomassie-stained gels. This confirms the earlier conclusion that pMMB503EH is not an expression vector appropriate for *E. coli* (see Chapter 4.6) and if overexpression in *E. coli* is desired, then a different vector should be used. For this a new strategy was developed to express FccA56 in *E. coli*, by using the pT7-7 system.

5.4 Cloning of the *fccA56* gene into the pT7-7 vector and protein overexpression in *E. coli* HMS174

The vector used for FccA56 expression in *E. coli* was pT7-7, which contains an IPTG-inducible T7 promoter. The 1.5 kb *Bam*HI/*Pst*I fragment was cloned into pT7-7 cut with the same enzymes, to form pDA4-8. In order to maximise the expression, the 24 bp fragment that lies between the first start codon, already incorporated in the plasmid sequence (at the *Nde*I site), and the FccA56 start codon was eliminated by restriction digestion with *Nde*I and *Bam*HI, to form pDA4-9. The construct was transferred to a variety of *E. coli* strains (HMS174, BL21, BL21(DE3), JM109(DE3), B834) and levels of expression determined. The cells were grown with good aeration until $OD_{600nm} \sim 0.5$ and were induced with IPTG at concentrations ranging from 0.01-1 mM for four hours. The cells were harvested and the pellets were resuspended in 0.2 ml 10 mM phosphate buffer pH 7. Total cell extracts were run on a 10 % SDS-PAGE gel which was subsequently stained with Coomassie-Blue and destained in destaining solution.

Overexpression of FccA56 was only observed for *E. coli* HMS174, as shown in Fig. 5.7. In all the other strains, no overexpressed band was observed in the gel.

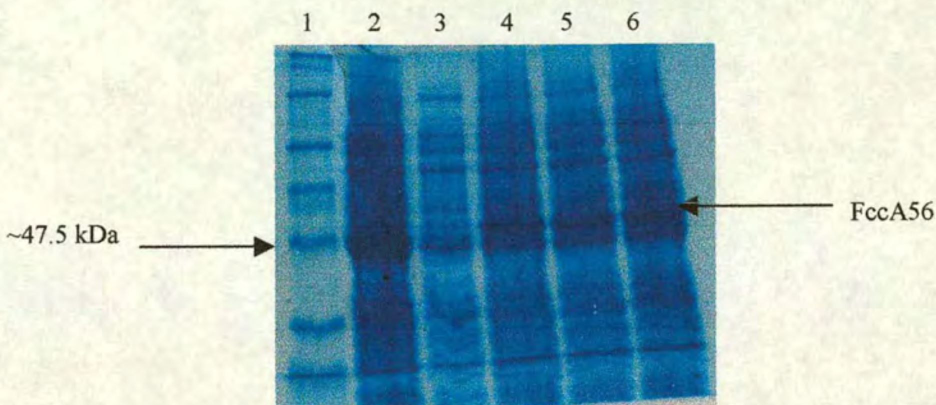

Fig 5.7 Coomassie-Blue stained 10 % SDS-PAGE gel of FccA56 overexpression in *E. coli* HMS174 cells.

Lane 1- Broad range protein marker (NEB); Lane 2- uninduced cells; Lane 3- 0.01 mM IPTG; Lane 4- 0.1 mM IPTG; Lane 5- 0.5 mM IPTG; Lane 6- 1 mM IPTG

In order to determine whether the protein is soluble or not, the same samples were sonicated and both the pellet and the supernatant were run on a 10 % SDS-PAGE gel. After electrophoresis, 80 % of the protein was found in the soluble fraction.

5.5 Purification of FccA56 expressed in *E.coli* HMS174

For protein purification, a 5 litre culture was used, grown under the same conditions as described in Chapter 5.4. After harvesting, the pellet was resuspended in 10 mM phosphate buffer pH 7 supplemented with protease inhibitors (10 mM benzamidine and 20 mM PMSF) and sonicated for 1 min / gram of pellet. During

purification trials, it was found that the protein lost its flavin prosthetic group. From the early stages of purification yellow fractions were collected which had an absorption maximum at 450 nm (corresponding to the flavin group), but no ~52 kDa protein was associated with this. To minimise loss of flavin, the growth conditions were altered; after induction with 100 μM IPTG, the temperature was decreased from 37 °C to 30 °C, the shaker speed decreased from 160 to 70 rpm and the cells were grown overnight in TB medium instead of LB.

After trying different chromatographic columns, the following combination yielded a ~95 % pure FccA56. After sonication and centrifugation, the soluble fraction was loaded onto a 20 ml DEAE-Sephacel column equilibrated in 10 mM phosphate buffer pH 7. The flow-through was collected and loaded on a 20 ml hydroxyapatite column equilibrated in the same buffer. A compact yellow band was observed at the top of the column. Protein was eluted with an ammonium sulfate gradient of 0-6 % (w/v). The yellow band was eluted at 5 % $(NH_4)_2SO_4$. These fractions contained ~95 % pure protein with an apparent molecular weight of about 52 kDa (see Fig 5.8). The characteristic UV-visible spectrum obtained confirmed that this was a flavoprotein with a typical absorption maximum at 450 nm (see Fig 5.9).

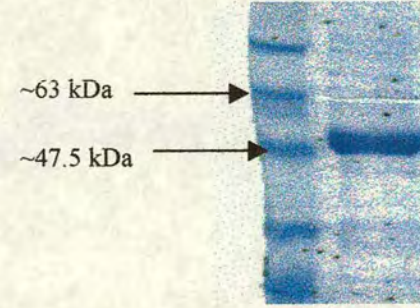

Fig 5.8 10 % SDS-PAGE gel of the purified FccA56

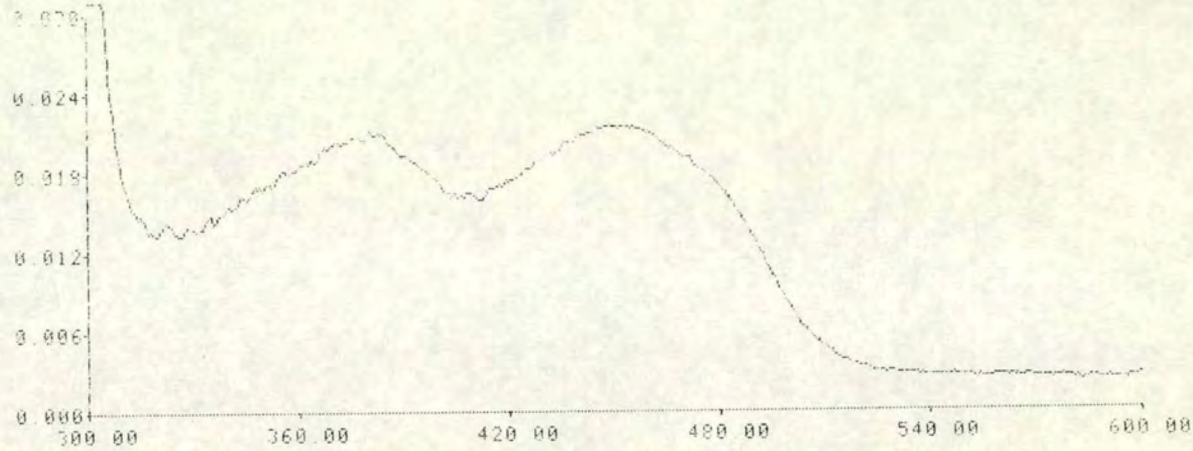

Fig 5.9 UV/visible absorption spectrum of pure FccA56

Considering that FccA56 has a theoretical extinction coefficient of approximately 410000 M^{-1} cm^{-1} at 280 nm (ProtParam Tool programme) and the FAD group around 11000 M^{-1} cm^{-1}, it was estimated that only about 7 % of the protein contained bound flavin. Hoever, if all purification steps were carried out as rapidly as possible on the same day, the percentage of retained flavin increased to 12 %.

In order to minimise the loss of the flavin group FccA56 could be expressed and purified as a His-tagged protein. This would allow a one-step purification, when columns charged with Ni^{2+} ions would selectively retain the protein via the six-histidine residues exposed on the surface of the protein. The *fccA56* gene was amplified using two different sets of primers: A56L/A56-His (see Materials and Methods) designed to generate a DNA fragment coding for a double arginine signal sequence and six histidine

residues at the C-terminal end, and A56-no ss/A56-His which would amplify a fragment without signal sequence (cytoplasmic localisation) and with six histidine residues. Both fragments were cloned into the pGEM-T vector to form pDA4-81 and pDA4-84 respectively. The second construct (His-tagged and without signal sequence) was cloned into pT7-7 to form pDA4-85 and it was expressed in *E. coli* HMS174 cells under the same conditions as described for the pDA4-9 construct. Although the protein was expressed at the same levels as before, no protein bound to the nickel column. After sonication and centrifugation, prior to loading onto the Ni^{2+} column, the soluble fraction from 2 litres of culture was filtered to avoid the column clogging, as described in Materials and Methods. It is possible that the protein misfolded and the His-tag was buried into the polypeptide and it did not bind to the Ni^{2+} column. The His-tagged protein would have allowed a shorter purification procedure and the isolation of a protein with a higher flavin content. The failure of this approach made us use the initial pDA4-9 construct for the biochemical characterisation of the FccA56 flavoprotein. For all the experiments, the values were corrected for the flavin content.

5.6 FccA56 kinetic characterisation

5.6.1 Enzyme assay

Based on the model proposed before (see Chapter 5.1.2) FccA56 was assayed for its ability to reduce various monocarboxylic substrates (see Table 5.1). The method used was similar to that for fumarate reduction by Fcc_3 under anaerobic conditions (Doherty *et al.*, 2000). The basis of the assay is the fumarate dependent reoxidation of reduced methyl viologen. When reduced by sodium dithionate, methyl viologen absorbs in the

blue region of the visible spectrum. Reoxidation causes methyl viologen to become colourless and the extent of this conversion is monitored at 600 nm. If any oxygen is present, this will act as an electron acceptor and methyl viologen will be oxidised. To ensure complete absence of oxygen, all solutions were degassed and the work was carried out in an anaerobic glove box as described in Materials and Methods. Background traces were monitored to ensure no oxygen was present before the addition of 200 mM substrate. The reaction was initiated by the addition of 5 μM active enzyme. The change in the absorbance was measured as a function of time. The observed rate constant was calculated using equation:

$$k_{obs} = \Delta_{abs} / t \; \varepsilon \; [E]$$

k_{obs} = the observed rate constant

Δabs = the measured change in absorbance
t = time (seconds)
ε = methyl viologen extinction coefficient
[E] = FccA56 concentration, corrected for the FAD content

Equation 5.1 Determination of rate constant

The ability of FccA56 to reduce the potential substrates listed in Table 5.1 was observed at pH 7.2 in a completely anaerobic environment. With the exception of fumarate, no activity was detected with any of the compounds.

The measured K_M for fumarate was 220 μM which is ten fold higher than the value measured with Fcc_3 (25 μM; Doherty *et al,* 2000). Coupled with the low k_{cat} value 0.15 s^{-1} (509 s^{-1} for Fcc_3) this indicates that fumarate is unlikely to be a physiological relevant

Table 5.2 The structure of potential aerobic substrates

Aspartic acid

Asparagine

Alanine

Malic acid

Histidine

Serine

Arginine

Leucine

Succinic acid

substrate for FccA56 (see Fig 5.10). The addition of FAD or cytochrome c_3 into the assay solution (to create an Fcc_3-like complex) did not improve the reduction rates.

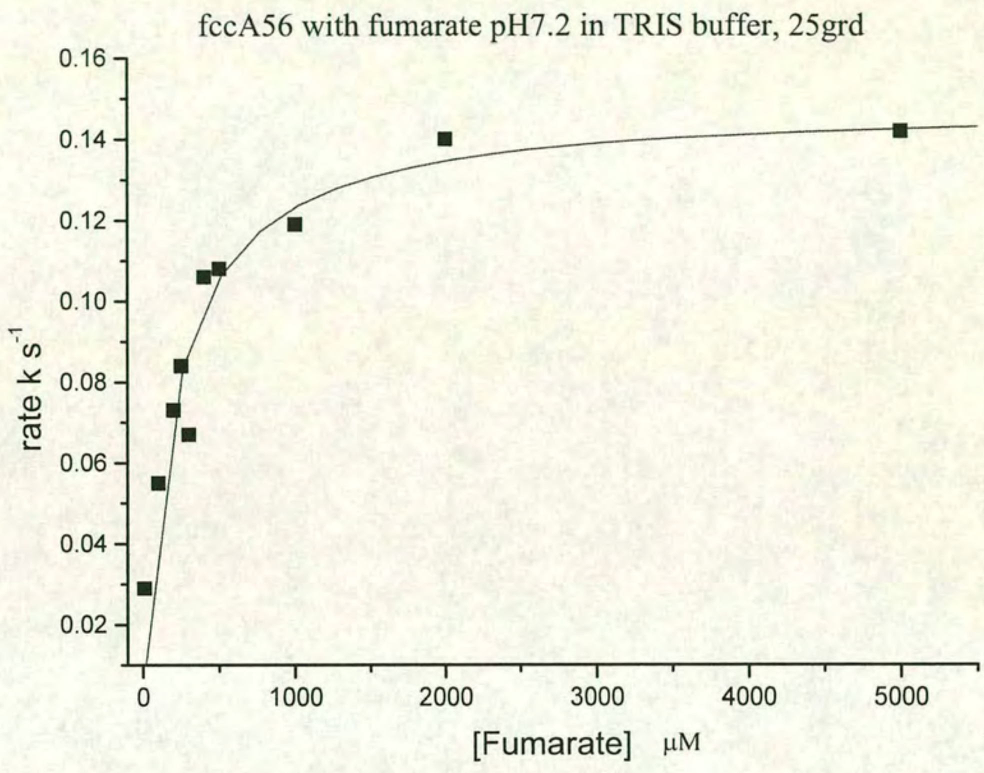

Fig 5.10 Michaelis plot for fumarate reduction by FccA56 at pH 7.2

In Fcc_3 the active site is at the interface between the flavin-binding and clamp domain (Taylor *et al.*, 1999) and domain movement is important. Opening is necessary for the substrate access to the active site and closure is essential for catalysis (Reid *et al.*,

2000). Although some activity is observed for fumarate, it could be speculated that the active site is formed and binds the substrate but the substrate is not reduced because the protein is in an open conformation (which would explain the loss of the flavin group during purification). The presence of the cytochrome FccB56 and probably of the Hal56 may be required to achieve a catalytically active complex. This hypothesis could be checked if forms of the Fcc56 complex (FccA56 + FccB56; FccA56 + FccB56 + Hal56) are isolated and characterised.

5.6.2 Oxidation assay

Given the results obtained under anaerobic conditions, the ability of FccA56 to catalyse the reverse reaction, the oxidation of different substrates was assayed (see Table 5.2).

These substrates were tested under aerobic conditions with dichloroindophenol (DCIP) as the electron acceptor, as described in Materials and Methods, but no activity was detected with any of them. All these kinetic studies would need to be repeated once the FccA56 + FccB56 and FccA56 + FccB + Hal56 are isolated, for the reasons described before.

5.7 Potentiometric titration

In order to determine the flavin reduction potential, FccA56 was titrated with sodium dithionite and the change of the optical absorption spectrum was measured at a range of electrode potentials (see Fig 5.11).

Table 5.1 Structure of potential substrates for FccA56 in anaerobic conditions

fumarate

acrylic acid

crotonic acid

tiglic acid

methacrylic acid

3,3 dimethylacrylic acid

mesaconic acid

trans−cinamic acid

caffeic acid

ferulic acid

benzoic acid

p−coumaric acid

dihidroxybenzoic acid

trans−3(3−pyridil)acrylic acid

urocanic acid

3−β−indoleacrylic acid

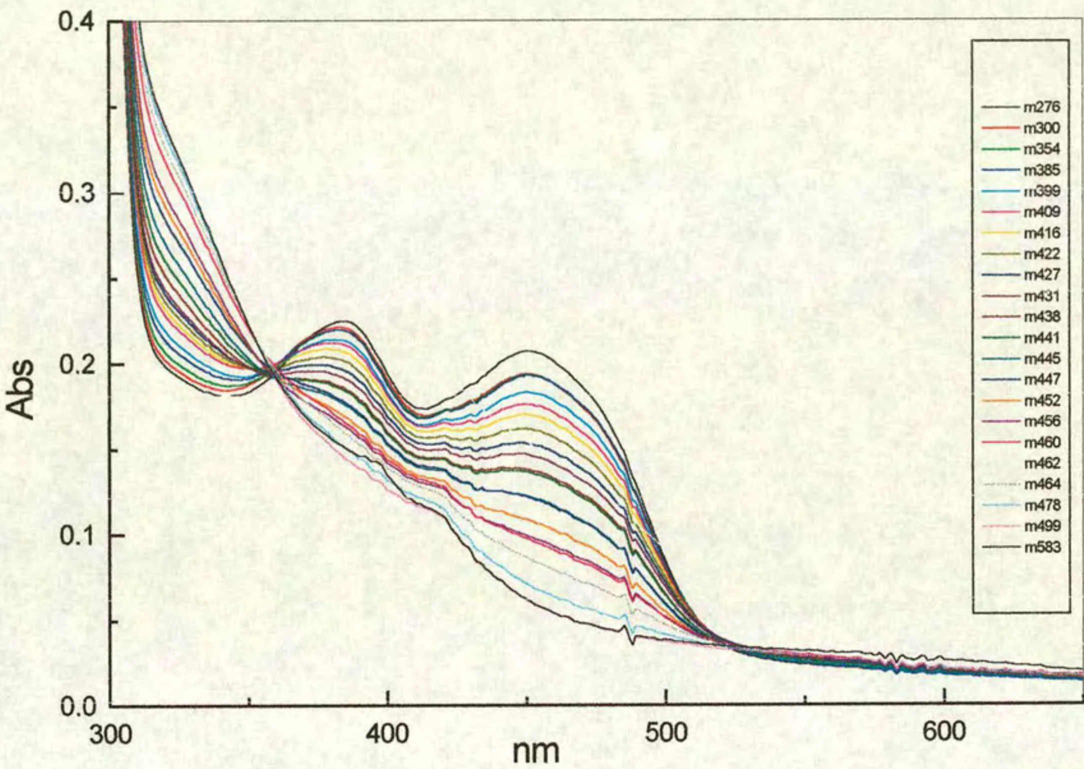

Fig 5.11 Succesive spectra used to determine the FAD potential

The obtained A_{450} values were plotted and the curve was fitted to a two electron transfer Nernst equation (see Fig 5.12). The measured potentials were –212 and –190 mV.

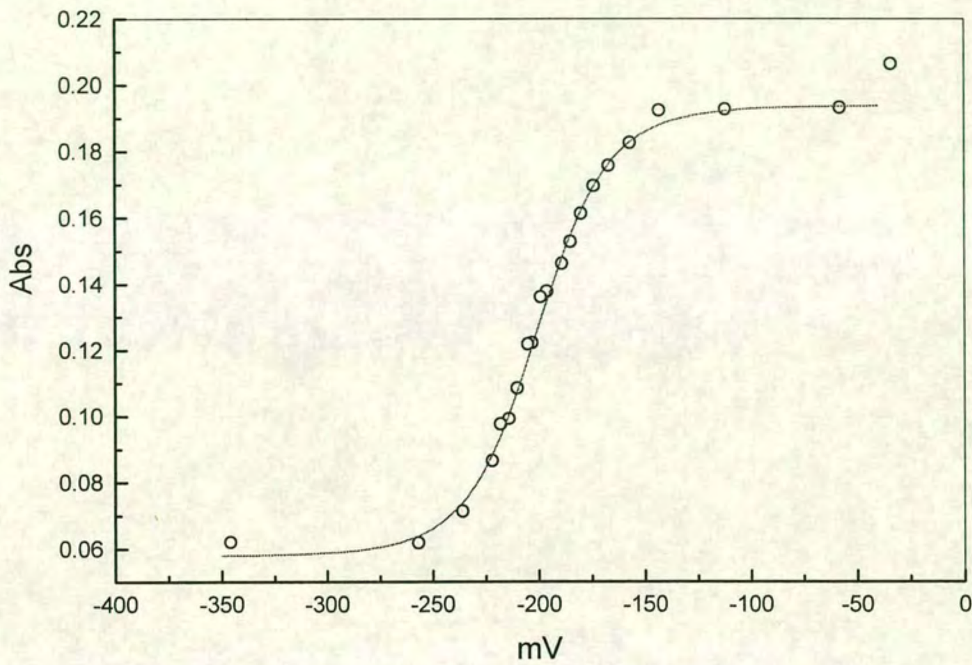

Fig 5.12 Plot of absorbance at 450 nm against the measured potential for FccA56

5.8 Isolation of *fccA56-fccB56* and *fccA56-fccB56-hal* genes

As mentioned in Chapter 5.6.1, the loss of the flavin group during purification and the lack of enzymatic activity with any of the tested substrates indicated that FccA56 exhibits low activity in the absence of FccB56 and possibly Hal56 as well. To test this, different forms of the complex would need to be isolated and characterised.

The *fccA56-fccB56* genes were PCR-amplified using primers A56L and B56R and the product cloned into the pGEM-T vector, to form pDA6. For protein

overexpression, the *fccA56-fccB56* combination was cloned as a *Bam*HI fragment into the pT7-7 vector (pDA6-2). Considering the problems that usually arise when *c*-type cytochromes are expressed in *E. coli* (see Chapter IV), the construct was co-expressed with plasmid pEC86 (contains the *ccm* genes involved in cytochrome *c* biogenesis; see Chapter IV) in a variety of *E. coli* strains (see Fig 5.12). The flavoprotein expression was very good in HMS174 cells (see Fig 5.13) but the amount of *c*-type cytochrome (as seen on a heme stained gel) was low. However, late log phase induction increased the amount of FccB56.

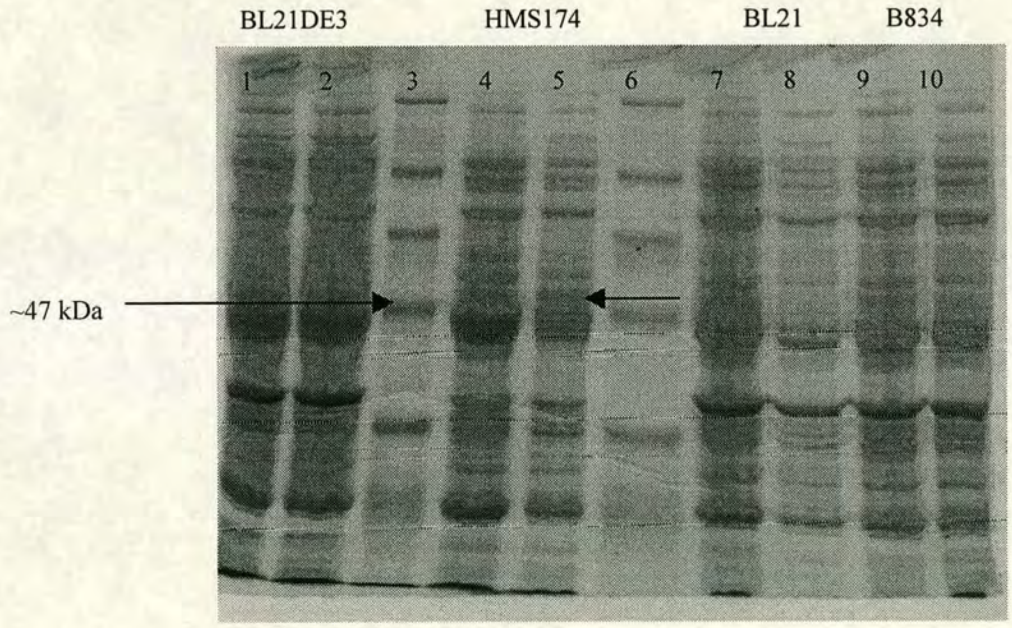

Fig 5.13 FccA56 overexpression in the FccA56-FccB56 complex with 1 mM IPTG and 4 hours induction

Lanes 3, 6- Broad-range protein marker (NEB); Lanes 1, 4, 7, 9- uninduced cells; Lanes 2, 5, 8, 10- induced cells

The *hal56* gene was amplified alone, using Hal56(L) / Hal56(R) primers and the product cloned into the pGEM-T vector (to form pDA7) and as part of the three-gene cluster *fccA56-fccB56-hal56* and the 3.8 kb product cloned into pGEM-T (pDA8). Further work would involve the expression of pDA6 and pDA8 in either *E. coli* (by co-expression with pEC86 plasmid) or *Shewanella* MR-1.

Chapter VI Gene Disruption

6.1 Introduction to gene disruption

To determine the function of a gene the construction and characterisation of mutants is essential. There are several approaches available to generate mutants: random mutagenesis using chemicals or transposons or gene replacement of previously cloned DNA that has been manipulated by introducing insertions or deletions.

Transposons are DNA fragments that can move to new locations in DNA molecules. Those commonly used encode resistance to the aminoglycoside antibiotics kanamycin and neomycin. Transposons are highly transposable when introduced into a cell. However, reversion by precise excision of the inserted transposon occurs at very low frequencies. Insertion of a transposon may occur within a gene, resulting in complete loss of gene function. If the gene is essential, gene inactivation is associated with changes in phenotype.

An example of successful transposon mutagenesis in *Shewanella* sp, led to the identification of the *cym*A gene, a tetraheme cytochrome *c* required for reduction of Fe(III), fumarate and nitrate but not TMAO (Myers & Myers, 1997a).

Chemical mutagenesis can induce two major classes of mutations:

i. point mutations, in which one base pair replaces another

ii. insertion/deletion mutations

Point mutations can result from the treatment of an organism with base analogues or substances that chemically alter bases, for example 5-bromouracil or 2-aminopurine. Insertion/deletion mutations may arise from the treatment of DNA with intercalating agents such as acridine orange or proflavine. The distance between two consecutive base pairs is doubled by the intercalation of such a molecule between them. The

replication of such distorted DNA occasionally results in the insertion or deletion of one or more nucleotides in the newly synthesised polynucleotide. One of these substances, the alkylating agent ethyl methanesulfonate was used to create random mutants in *Shewanella* 200. By plating the resulting clones on minimal medium supplemented with different electron acceptors, mutant strains deficient in Fe(III) (DiChristina & DeLong, 1994) and Mn(IV) (Burnes *et al*., 1998) reductase activity were isolated.

The main disadvantages of these methods are the large number of colonies that need to be screened in order to identify the desired phenotype and the tediousness of identifying the exact location of the mutation.

When site specific inactivation is required, gene replacement can be used; cloned DNA fragments are manipulated by introducing insertions, deletions or base replacement. The plasmid used for gene replacement needs to be a suicide vector (unable to replicate in the bacterial strain where the modifications occur) and mobilizable (contains RP4 *mob* gene).

There are two ways of creating deletions:

i. marked deletions, when an antibiotic cassette (or other selectable marker) is inserted into the target gene
ii. unmarked deletions, when the gene is cloned after manipulation, into a plasmid containing a marker that enables single and a double cross-over to be selectable.

6.2. Gene disruption in *Shewanella* spp.

Replication of a plasmid is regulated at a stage of initiation that occurs at a specific site called the origin of replication (*ori*). Most vectors in current use carry a replicon derived from the plasmid pMB1 (Hersfield *et al.*, 1974) or p15A (Chang & Cohen, 1978) and lack a gene (*mob*) required for mobilization. They are therefore incapable of directing their own conjugal transfer from one bacterium to another.

Previous work in *Shewanella* MR-1 involved the use of plasmids derived from pACYC184 which carry a *p15A* origin of replication. Saffarini & Nealson (1993) constructed an *etr*A mutation (analogue of the *E. coli fnr* gene) in pACYC184 and tested it for the ability to grow anaerobically with various terminal electron acceptors. The *etr*A mutants were deficient in growth on nitrite, thiosulfate, sulphite, TMAO, DMSO, Fe(III) and fumarate, suggesting that EtrA is involved in the regulation of the corresponding reductase genes. The mutants were positive for reduction of nitrate and Mn(IV) indicating that EtrA is not involved in the regulation of these systems.

Similarly, the role of fumarate reductase from *S. frigidimarina* NCIMB400 was examined by constructing a null mutation (Gordon *et al.*, 1998). Part of the *fccA* coding sequence was replaced by a kanamycin resistance cassette and the disrupted gene cloned into pJQ200KS, a suicide plasmid that contains *sac*B, a conditionally lethal gene, and a *p15* origin. Analysis of mutant strains demonstrated that the cells could not grow anaerobically with fumarate as the terminal electron acceptor, but growth patterns with nitrate, TMAO, Mn(IV), thiosulfate and sulfite were unaffected.

At the time this project was started a study of plasmids and their replication in *Shewanella* MR-1 had just been published. Based on the study by Saffarini &

Nealson (1993), Myers & Myers (1997c) tried to use pACYC184, a tetracycline resistance plasmid, in gene replacement. After transfer of the plasmid into MR-1 through electroporation, numerous transformants were obtained. It was demonstrated that all of the clones maintained the plasmid (Myers & Myers, 1997c). Introduction of the tetracycline resistance plasmid pBR322 (*pMB*1 origin) into *Shewanella* MR-1 by electroporation failed to yield any Tc^R transformants. The difference between these two plasmids is at the origin of replication level. The conclusion of the paper was that "*plasmids with pMB1 origin of replication (pBR322) should be suitable vectors for gene replacement in Shewanella MR1, whereas those with the p15A origin (pACYC184) are not suitable*".

On this basis, a plasmid with a pMB1 origin was chosen for the purpose of generating deletion strains in *Shewanella* MR-1 of *cyc129*, *cyc202* and *fccA56*. All DNA manipulations were carried out in pGEM-T, unless otherwise stated.

5.3 Unmarked deletions

The presence of antibiotic resistance markers can result in some limitations in complementation analysis or in the construction of strains with multiple mutations. A suicide vector carrying a conditional lethal gene that would discriminate between the integration of the vector and double recombination events would overcome this problem. An ideal candidate is the sucrose-inducible *sac*B of *Bacillus subtilis*. The *sac*B gene encodes levansucrase (sucrose:2,6 β-D-fructan 6 β-D-fructosyl transferase) an enzyme secreted in culture medium by *B. subtilis* after induction by sucrose. The product of levansucrase in *E. coli, Agrobacterium tumefaciens* and

Rhizobium meliloti is lethal in the presence of 5 % sucrose, causing lysis within one hour or inhibition of growth (Gay *et al*., 1983).

Considering the advantages of generating unmarked multiple deletions in the same strain and the Myers & Myers study (1997c) on plasmid replication in *Shewanella* MR-1, a new strategy was developed to construct unmarked deletions (Fig 6.1). This requires four steps:

i. construction of the unmarked deletions in pGEM-T (*pMB1* origin) and cloning of the *sac*B+*tra*J+Gm^R fragment from pJQ200KS
ii. introduction by single homologous recombination of the plasmid into the *Shewanella* MR-1 recipient and selection for the Gm^R
iii. second homologous cross-over with the selection for sucrose resistance (loss or inactivation of the *sac*B) and gentamicin sensitivity (loss of the plasmid)
iv. distinction between the two possible double recombinants: restored wild type fragment in which the two cross-overs occur in the same flanking region of the deletion and unmarked deletion mutants resulting from cross-overs in opposite sides of the deletion. Phenotypic screening, PCR amplification or Southern blot hybridization could then be performed to identify a recombinant strain that had transferred the deletion to the chromosome.

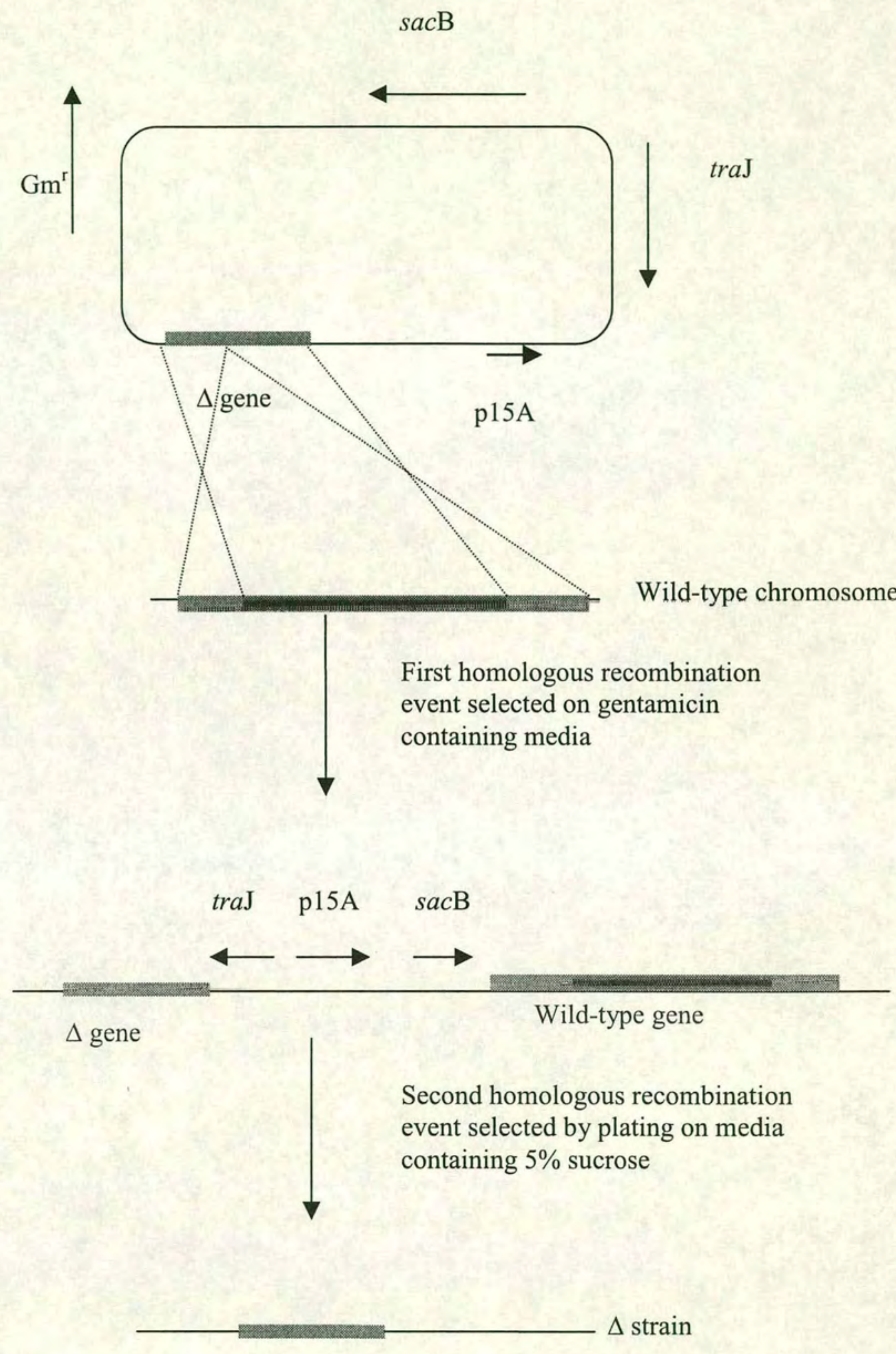

Fig 6.1 Scheme of the expected mechanism for replacement by homologous recombination of an inactivated chromosomal gene for a gene with a deletion made *in vitro*.

6.3.1 Construction of the unmarked deletions in a plasmid with *pMB1* origin of replication for *cyc129*, *cyc202* and *fccA56*

Previously cloned PCR products in pGEM-T (*pMB1* origin) corresponding to intact *cyc129*, *cyc202* and *fccA56* were used for gene disruption, by eliminating part of the encoding sequence.

For *cyc129*, plasmid pDA2 (5.2 kb) was digested with *Psh*AI and *Msc*I. The 4.3 kb blunt-ended fragment was purified and religated to form pDA2-2. A 4.2 kb *Sma*I/*Ssp*I fragment from pJQ200KS containing the gentamicin cassette, *sac*B gene and transfer *tra*J gene was cloned into pDA2-2 cut with *Xmn*I to form pDA2-3.

The disrupted *202* and *fccA56* genes were generated in a similar manner. pDA3 containing intact *202* gene was cut with *Bgl*II/*Eco*RI, 905 bp eliminated, the fragment end-filled, and the 4.2 kb fragment religated to form pDA3-2. The same 4.2 kb *Sma*I/SspI fragment from pJQ200KS as described above was cloned into pDA3-2 to form pDA3-3.

For *fccA56*, 813 bp were eliminated by restriction digest with *Bsu*36I/*Xba*I; the 3.6 kb fragment end-filled and religated to form pDA4-2. Cloning of the 4.2 *Sma*I/*Ssp*I fragment into the pDA4-2/*Sca*I site generated pDA4-3.

Prior to conjugation, all pDAn-3 plasmids were transferred to the *E. coli* S-17 strain which enables conjugative transfer. The transconjugants were selected for gentamicin resistance in the first round and then on sucrose. The colonies that survived sucrose selection were tested for their sensitivity to gentamicin. Although

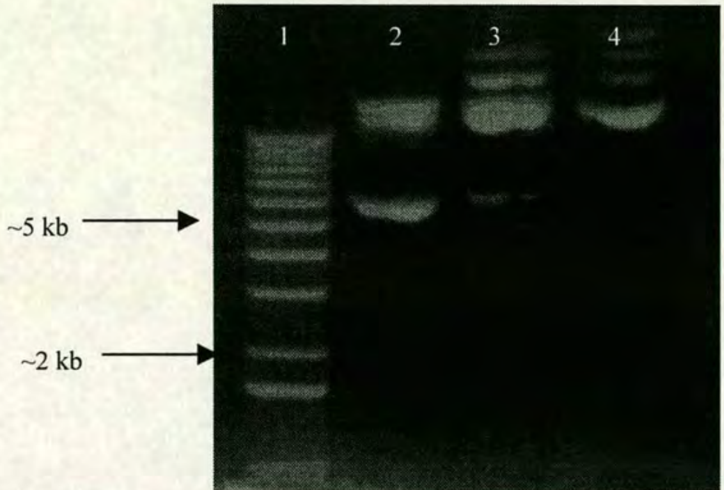

Fig. 6.2 Plasmid DNA isolated from colonies resulting from conjugal transfer of pDA2-3, pDA3-3 and pDA4-3 to *Shewanella* MR-1.
Lane 1-1kb DNA molecular marker (GibcoBRL). Lanes 2,3,4 -plasmid DNA from Δ*129*, Δ*202* and Δ*fccA56* clones, respectively

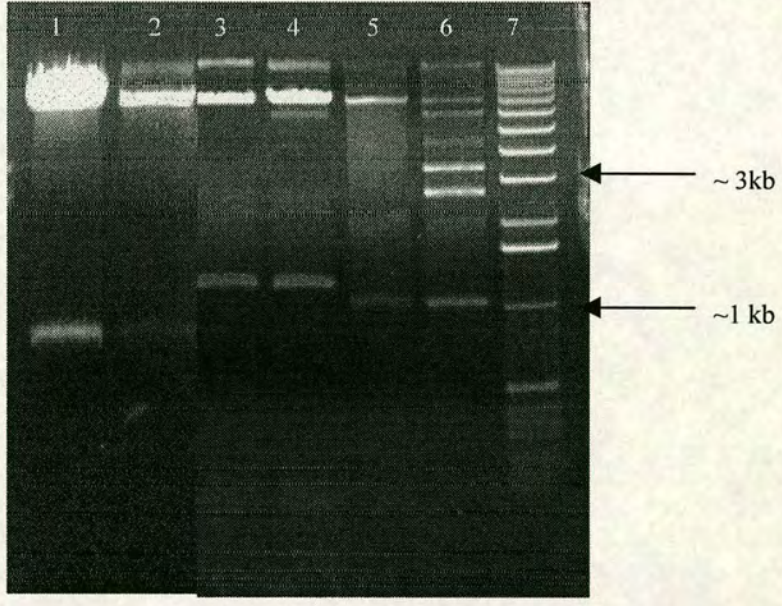

Fig. 6.3 Restriction digests performed on plasmid DNA isolated from a few of the transconjugants obtained.
Lanes 1+2 Δ*fccA56* plasmid DNA cut with *Bam*HI/*Sma*I; lanes 3+4 Δ*129* plasmid DNA cut with *Eco*RI/*Bam*HI; lanes 5+6 Δ*202* plasmid DNA cut with *Bam*HI/*Sma*I; Lane 7- 1 kb DNA marker (GibcoBRL)

hundreds of colonies were screened, no gentamicin sensitive/sucrose resistant colonies were isolated. A few colonies were isolated and further analysed. Overnight cultures were used to isolate genomic and plasmid DNA as described in "Materials and Methods". Contrary to the conclusion of Myers & Myers (1997c), plasmid DNA was isolated from *Shewanella* MR-1, which indicated that the plasmid was maintained as a free replicon (Fig 6.2). Restriction digests performed on these plasmids confirmed that they were the original plasmids transferred by conjugation (Fig 6.3).

6.3.2 Transfer of the deleted genes into pJQ200KS

The difference in results obtained here compared with those reported by Myers & Myers (1997c) raises a question mark over the origins of replication required for successful gene replacement in *Shewanella* MR-1. As mentioned before (see Chapter 6.2), a fumarate reductase deleted strain in *Shewanella frigidimarina* NCIMB400 had been successfully generated using pJQ200KS (*p15A* origin). In order to exclude the possibility of a similar system for *Shewanella* MR-1, all disrupted genes were cloned into pJQ200KS (*p15A* origin) and transferred via conjugation to *Shewanella* MR-1. Unfortunately, the plasmids replicated as before, consistent with the results reported by Myers & Myers (1997c). If homologous recombination is highly efficient, a mutant strain could be isolated even when the plasmid replicates. Despite numerous trials and intensive screening by replica plating and colony PCR, no double cross-over transconjugants were isolated.

6.3.3 Marked deletions in pEP185.2

Although unmarked deletions provide a useful tool in analysing multiple gene disruptions in the same strain, our main concern was the absence of a suitable plasmid that could be used for null mutation constructs in *Shewanella* MR-1.

A third origin of replication derived from the 38 kb self-transmissible R6K plasmid was used. R6K replication requires a plasmid-encoded protein, designated π, for initiation of DNA replication (Kolter *et al.,* 1978). The π protein has been implicated in negative regulation of plasmid replication, providing a level of replication control specific to R6K (MacAllister *et al.,* 1991). Replication of plasmids derived from R6K is restricted to strains that carry a derivative of plasmid RP4 and the gene that encodes for π protein is integrated in the bacterial chromosome.

pEP185.2 (kindly provided by Virginia Miller) has the 420 bp fragment containing the R6K origin, the multiple cloning site derived from pBluescript and the *mob* from RP4 (Miller & Mekalanos, 1988, Pepe & Miller, 1993, Kinder *et al.*, 1993). The replication of this plasmid is restricted to *pir* strains. SM10 λ*pir* (or S-17 λ*pir*) can mobilise pEP185.2 and can provide conjugative functions *in trans* to the *mob* site on pEP185.2 but is rarely transferred to the recipient strain.

To construct marked mutations in the *cyc129*, *cyc202* and *fccA56* genes, the kanamycin resistance cassette from pRS552 (Simons *et al.,* 1987) was extracted as a 1.4 kb *Stu*I fragment and cloned into previously modified plasmids. Plasmids pDA2-2 and pDA3-2 that contain the disrupted *cyc129* and *cyc202* genes (see Chapter 6.3.1) were cut with *Pml*I and ligated with the Km^R cassette, to form pDA2-6 and pDA3-6, respectively. For *fccA56* disruption, pDA4 was cut with *Pml*I/*Bsu*36I, the

fragment blunt ended and ligated with Km^R cloned to form pDA4-6. All modified genes were cloned into pEP185.2 as *ApaI/PstI* fragments. The resulting plasmids, pDA2-7, pDA3-7 and pDA4-7 were transferred to *Shewanella* MR-1 via conjugation and the transconjugants analysed by colony PCR to determine the presence of the disrupted copy in the chromosome.

Although the plasmids did not replicate in the *Shewanella* host (no plasmid DNA was isolated), no cross-over had occurred. Only bands corresponding to the native genes were generated by colony PCR (Fig. 6.4).

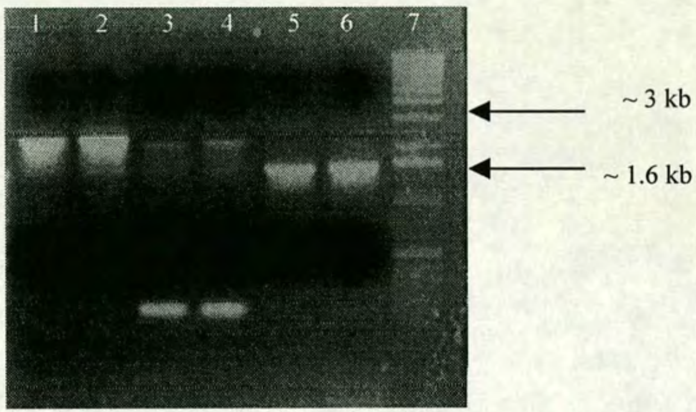

Fig. 6.4 Colony PCR on rifampicin+kanamycin resistant cells resulting from the transfer of pDA2-7, pDA3-7 and pDA4-7 respectively to *Shewanella* MR-1.
5μl lysate was added to the PCR mix containing 12.5mM of each dNTP, 10 pmol of each primer and 1U Taq DNA polymerase. The cycling parameters were as follows: 1 min at 94 °C, 25 cycles of 30 sec at 94 °C, 30 sec at 40 °C and 45 sec at 72 °C and 3 min at 72 °C.
Lanes 1+2-Δ*129* with 129L/129R primers; lanes 3+4- Δ*202* with 202L/202R primers; lanes 5+6-Δ*fccA56* with fccA56L/fccA56R primers; lane 7- 1kb DNA marker

The fact that all clones tested contain the intact gene and are kanamycin resistant raises two important questions:

i. is Km^R present in the chromosome or are the cells spontaneously kanamycin resistant?

ii. if kanamycin is present in the chromosome is it inserted at the same place in each clone or is it transposed to different locations?

Genomic DNA from four different, presumed deleted, *cyc129* clones (Δ*129*(?)) was isolated and each cut with *Hin*dIII (cuts in Km^R), *Sal*I or *Sac*I restriction enzymes. The DNA was transferred to a nylon membrane and probed with DIG-labelled Km^R from pRS551, as described in "Materials and Methods".

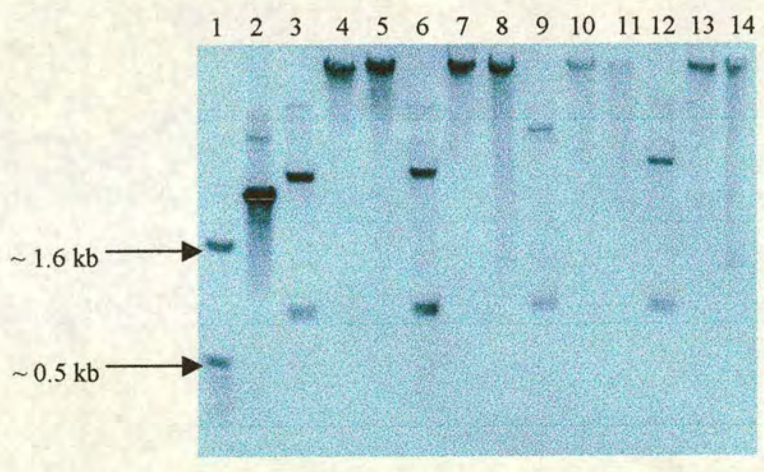

Fig. 6.5 Southern blotting for four randomly chosen clones; the kanamycin cassette from pRS552 was DIG-labelled as described in "Materials and methods".
Lane 1-1kb DNA marker (GibcoBRL); lane 2 *129* PCR product (positive control); lane 3, 6, 9, 12- genomic DNA cut with *Hind*III; lane 4, 7, 10, 13- genomic DNA cut with *Sal*I; lane 5, 8, 11, 14- genomic DNA cut with *Sac*I. The antibiotic cassette contains a restriction site for *Hin*dIII.
The different pattern exhibited by clone number 3 could be the result of a different orientation of the resistance into the gene during manipulation.

The results confirm the presence of a single copy of the Km^R in the chromosome. The different pattern exhibited by clone 3 could be due to the insertion

in a reverse orientation of the blunt ended Km^R into the gene (Fig. 6.5). The Southern blot does not indicate the location of the antibiotic resistance.

In order to determine this, inverse PCR was performed. For this it was necessary to know the size of the fragment that contained the antibiotic resistance, in order to establish the PCR parameters. Genomic DNA from the presumed deleted *cyc129* (Δ*129*(?)) clone 1 was digested with a variety of restriction enzymes, transferred to a nylon membrane and probed with DIG labelled-Km^R. A fragment of about 3 kb resulting from digestion with *Bsr*GI was identified (not shown).

For inverse PCR, genomic DNA from four clones was digested with *Bsr*GI. After inactivation of the enzyme, the fragments were religated with T4 DNA ligase as described in "Materials and Methods". 5 µl of the ligation mixture was used to perform a PCR reaction using primers REVKAN1(R) and REVKAN2(R). These anneal at the end of the antibiotic cassette and amplify in opposite directions (Fig 6.6). Considering that the fragment identified by Southern blotting was ~ 3 kb and the size of the Km^R is 1.4 kb, the expected size of the PCR product would be ~1.6 kb.

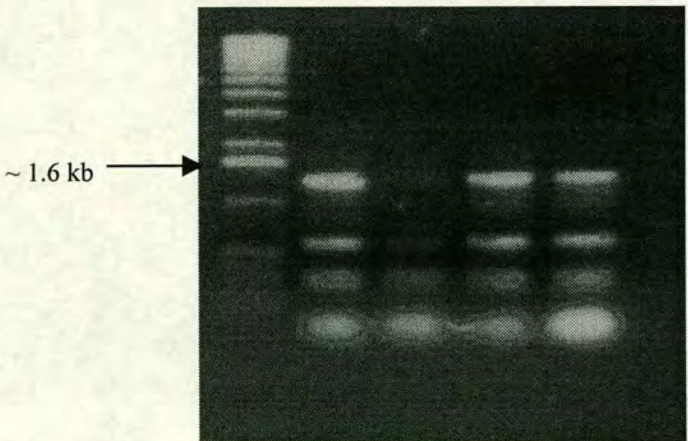

Fig. 6.6 Inverse PCR on four different genomic DNA isolated from rifampicin-kanamycin Δ129 resistant clones digested with *Bsr*GI; REVKAN1(R) / REVKAN2(R) primers were used. The cycling conditions were: 3 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 40 °C, 4 min at 68 °C and 5 min at 68 °C

The ~1.5 kb band obtained was gel purified (see "Materials and Methods") and cloned into pGEM-T to form 129-Km. Single stranded DNA was isolated and the insert sequenced using the (-40) primer. The sequence obtained was used for a Blast search against the *Shewanella* MR-1 genome. The *129-Km* gene gave a 100% match with a gene from *Shewanella* MR-1 of unknown function (see Fig. 6.7).

```
Score = 3108 (472.4 bits), Expect = 1.3e-135, P = 1.3e-135
 Identities = 646/667 (96%), Positives = 646/667 (96%), Strand = Minus / Plus

129-Km:    707 CGCCTTTGCAGGAAGGGATCGCTATTGACCCAAAAACAGGGGCGAGATTCATGAATGAAT 648
               ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288325 CGCCTTTGCAG-AAGGGATCGCTATTGACCCAAAAACAGGGGCGAGATTCATGAATGAAT 288383

129-Km:    647 TAGCAGACCGTCGAACTTCGTGCAGATGCGCAGTTGGCAGTGTTGGCCTCAGGTACGAAA 588
               ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288384 TAGCAGACCGTCGAACT-CGTGCAGATGCGCAGTTGGCAGTGTTGGCCTCAGGTACGAAA 288442

129-Km:    587 GAAAAACCTAATATGCCGTTTGTATTTTGTGGTGAGGCAACGGCAAATCATGCAGAAGGA 528
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288443 GAAAAACCTAATATGCCGTTTGTATTTTGTGGTGAGGCAACGGCAAATCATGCAGAAGGA 288502

129-Km:    527 TTTAAAGCGGCTTACCGTGATGGTGCAATTAAAAAGTCTGAAACGCTCGAAGAATTAGCC 468
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288503 TTTAAAGCGGCTTACCGTGATGGTGCAATTAAAAAGTCTGAAACGCTCGAAGAATTAGCC 288562

129-Km:    467 AAACGTTATGATGTTGATATTAATGCATTACAAAATTCAATAAATGAATGGAATGAGATT 408
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288563 AAACGTTATGATGTTGATATTAATGCATTACAAAATTCAATAAATGAATGGAATGAGATT 288622

129-Km:    407 GTTCAAGGTAAAGCAAAAGATCCTTTTAACAAACCTTTAGATGAAAAAACAATTTTAAAA 348
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288623 GTTCAAGGTAAAGCAAAAGATCCTTTTAACAAACCTTTAGATGAAAAAACAATTTTAAAA 288682

129-Km:     347 CCTCCATATTATTCAATCCGTTTGTCACCAAAATTACATTATTGCATGGGGGGGTTGCT 288
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288683 CCTCCATATTATTCAATCCGTTTGTCACCAAAATTACATTATTGCATGGGGGGGTTGCT 288742

129-Km:    287 ATTACGCCTAACGCAGAAGTCATTGACTCTAATACTTGCGAACCTATTTCAGGCTTATTT 228
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288743 ATTACGCCTAACGCAGAAGTCATTGACTCTAATACTTGCGAACCTATTTCAGGCTTATTT 288802

129-Km:    227 GCTGCTGGTGAAGTTACTGGTGGTACACATGGAATGGACCGATTAGGTGGTTGCTCTTCT 168
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288803 GCTGCTGGTGAAGTTACTGGTGGTACACATGGAATGGACCGATTAGGTGGTTGCTCTTCT 288862

129-Km:    167 ATTGATGGTCTCGTGTTTGGGCAAATTGCAGGTAATCAAGCTGCCATTAGAAAAGTGCCC 108
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288863 ATTGATGGTCTCGTGTTTGGGCAAATTGCAGGTAATCAAGCTGCCATTAGAAAAGTGTAA 288922

129-Km:    107 GGGGTGA 101
                 |||||
Sbjct: 288923 TAGGTGA 288929
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Fig. 6.7 Blast search of *129-Km* against *Shewanella* MR-1 genome (www.tigr.org)

As there is no similarity between the *cyc129* gene and the insertion site there is unlikely to have been a two-site cross-over. The same experiment was carried out for four, presumed deleted, *fccA56* (Δ*fccA56*(?)) clones. Inverse PCR yielded fragments of the same size as with 129-Km (Fig 6.8).

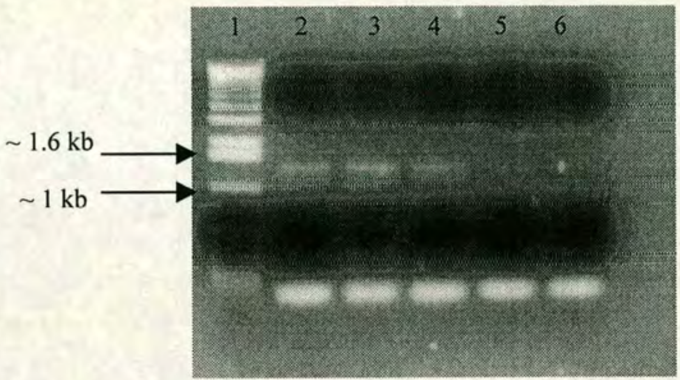

Fig 6.8 Inverse PCR on three different ΔfccA56 clones, using the same REVKAN1(R)/REVKAN2(R) primers, in the conditions described above.
Lane1- 1kb DNA marker (GibcoBRL); lane 2-4 ΔfccA56 PCR product; lane 5,6- *Shewanella* MR-1 genomic DNA cut with *Bsr*GI

Following sequencing and a Blast search, the same insertion site was found as for Δ*129*(?). Non-specific primer annealing is unlikely as a control carried out using genomic DNA from *Shewanella* MR-1 did not lead to any gene amplification.

The conclusion is that Km^R isolated from pRS551 is found in the same chromosomal site, regardless of the target gene.

Even though the same antibiotic resistance cassette was successfully used to inactivate the fumarate reductase from *S. frigidimarina* NCIMB400 (Gordon *et al.*, 1998), this proved unsuitable for *Shewanella* MR-1. The *Stu*I fragment from pRS551 contained, in addition to the Km^R insertion, repeats from Tn5 with a silent mutation in position 1442 of IS50L (Rothstein *et al.,* 1981) that eliminated the transposase

activity. In the *Shewanella* MR-1 environment these inverted repeats could have become active, creating a hot spot and in this way the whole fragment could be inserted into a specific site, irrespective to the target gene. To check this hypothesis, only the *ahp* gene from pSUP10141 was amplified by PCR using the Tn5Km(L) and Tn5Km(R) primers which introduce *Eco*RV sites at both ends. The resulting 900 bp fragment was cloned into pGEM-T (pCM51). pDA2 was digested with *Pml*I/*Hin*dIII, 513 bp eliminated, the fragment blunt ended and the Km^R inserted to form pCM2-10. Km^R was cloned in pDA3-2 digested with *Pml*I to form pCM3-10. For *fccA56* modification, pDA4 was cut with *Xba*I/*Pml*I, the fragment blunt-ended and ligated with the Km^R. The resulting plasmid was named pDA4-10.

The *cyc129, cyc202* and *fccA56*modified genes containing the new Km^R were extracted as *Sac*II/*Sal*I fragments and cloned into pEP185.2 cut with the same enzymes to form pDA2-11, pDA3-11 and pDA4-11 respectively. The plasmids were transferred to *Shewanella* MR-1 by conjugation and the resulting transconjugants checked for evidence of a double cross-over by colony PCR. For the Δ*129* and Δ*202* genes, the conjugation efficiency was high, with more than 200 colonies/plate, compared to Δ*fccA56* where no more than 20 colonies/plate were obtained. Around 40% of the Δ129 and Δ202 colonies were chloramphenicol sensitive (due to the loss of the pEP185.2 plasmid) and contained disrupted *cyc129* and *cyc202* genes respectively. For ΔfccA56, only 3 % of the obtained colonies were chloramphenicol sensitive and 2 % contained a complete replacement of the wild-type gene. (Fig. 6.9, 6.10, 6.11).

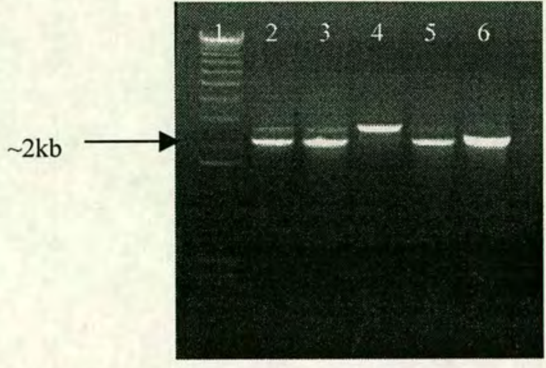

Fig 6.9 Colony PCR on 5 *Δ129* colonies Rf^r, Km^r, Cml^s obtained after the transfer of pDA2-11 to *Shewanella* MR-1. 5 μl of lysate were used to perform a 25 cycle PCR reaction: 30 sec at 94 °C, 30 sec at 40 °C, 2 min at 68 °C and a final extension step for 5 min at 68 °C.
Lane 1- 1 kb DNA marker (GibcoBRL); Lanes 2, 3, 5, 6- single cross-over recombination; Lane 4- double cross-over

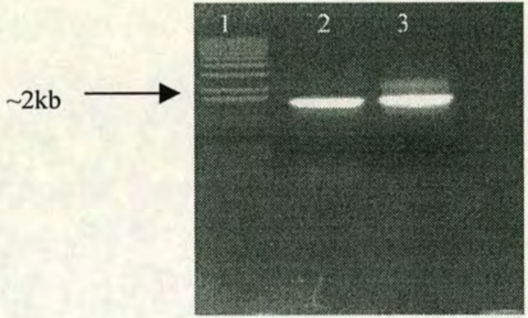

Fig. 6.10 Colony PCR on 2 *Δ202* Rf^r, Km^r, Cml^s colonies obtained after the transfer of pDA3-11 to *Shewanella* MR-1. PCR was performed as described above
Lane 1- 1 kb DNA marker (GibcoBRL); Lane 2- double cross-over; Lane 3- single cross-over

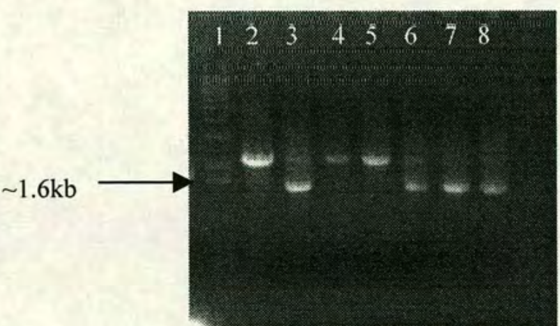

Fig. 6.11 Colony PCR on *ΔfccA56* Rf^r, Km^r, Cml^s colonies after the transfer of pDA4-11 to *Shewanella* MR-1. Conditions as described before
Lane 1- 1 kb DNA marker; Lanes 2, 4, 5- double cross-over; Lanes 3, 6, 7, 8- single cross-over

6.4 Phenotypic characterisation of the Δ129, Δ202 and ΔfccA56 deletion strains

Using wild-type *Shewanella* MR-1 as a control, the Δ129, Δ202 and ΔfccA56 deletion strains were tested for their abilities to grow anaerobically on minimal media supplemented with lactate as the sole carbon source and a variety of electron acceptors. Growth was tested on plates and liquid media.

6.4.1 Plate assay

Minimal medium (see Chapter 2.1.4) was supplemented with 15 mM lactate and different electron acceptors as listed in Table 6.1. Plates were spread with diluted overnight culture to give an estimated 100 cells per plate (assuming a density of 10^{10} – 10^{11} cells/ml for an overnight culture) and incubated under an H_2/CO_2 atmosphere (generated with an "Oxoid" gas generating system) at 23 °C. The ability to form colonies following 6-8 weeks incubation was then scored.

As shown in Table 6.1, growth patterns are the same for *Shewanella* MR-1 and the deletion strains. No growth was detected on minimal media plates supplemented with Fe(III), $NaNO_2$ or *trans*-cinnamic, indoleacrylic, hydroxycinnamic, acrylic, tiglic, methacrylic, senecioic, benzoic, caffeic and ferulic acids.

6.4.2 Liquid media assay

For growth on liquid media, 5 ml bijoux bottles were filled with the same minimal media as described above. Bottles were tightly sealed and incubated on a rotating platform at 23 °C for 3-4 weeks. The increase of carbon source

Table 6.1 Growth of the wild-type *Shewanella* MR-1, Δ129, Δ202 and ΔfccA56 strains using lactate as the carbon source and different electron acceptors

Electron acceptor	**MR-1 (wild-type)**	**Δ129**	**Δ202**	**ΔfccA56**
DMSO	✓	✓	✓	✓
TMAO	✓	✓	✓	✓
Fe(III) citrate	✗	✗	✗	✗
Fe(III) sulfate	✗	✗	✗	✗
Fe(III) chloride	✗	✗	✗	✗
Fumarate	✓	✓	✓	✓
$NaNO_2$	✗	✗	✗	✗
$NaNO_3$	✓	✓	✓	✓
Glycine	✓	✓	✓	✓
MnO_2	✓	✓	✓	✓
Na_2SO_3	✓	✓	✓	✓
Trans-cinnamic acid	✗	✗	✗	✗
Trans 3-pyridyl acrylic acid	✓	✓	✓	✓
Urocanic acid	✓	✓	✓	✓
Indoleacrylic acid	✗	✗	✗	✗
Hydroxycinnamic acid	✗	✗	✗	✗
Dihydroxybenzoic acid	✗	✗	✗	✗
Dimethylfumarate	✓	✓	✓	✓

Mesaconic acid	✓	✓	✓	✓
Acrylic acid	✗	✗	✗	✗
Crotonic acid	✓	✓	✓	✓
Tiglic acid	✗	✗	✗	✗
Methacrylic acid	✗	✗	✗	✗
Senecioic acid	✗	✗	✗	✗
Benzoic acid	✗	✗	✗	✗
Caffeic acid	✗	✗	✗	✗
Ferulic acid	✗	✗	✗	✗

concentration from 15 to 150 mM accelerated the growth rate and after 10 days a "growth / no growth" diagnostic test could be applied.

The results confirmed those obtained on the plate assays with the exception of the Δ202 deletion strain. After 4 weeks of incubation in the 5-25 mM MnO_2 medium, growth of all four strains was identical. When the MnO_2 concentration was increased to 30 mM, manganese reduction by the Δ202 strain was significantly slower than for the other three. This was evident after only a few days of growth. With this strain aggregated MnO_2 was observed at the bottom of the bottles, whereas the other three strains completely reduced the MnO_2 available. This observation was confirmed by repeating the experiment two more times. The significance of this result is uncertain. This phenotype can not be associated with a manganese reduction deficiency, as the deletion strain exhibits the same growth patterns as the wild-type strain at 5-25 mM MnO_2. (As MnO_2 is insoluble in water, the concentrations are approximated).

Chapter VII Discussion

Shewanella spp are a group of Gram-negative, facultatively anaerobic bacteria that populate a variety of habitats, from fresh water (Myers & Nealson, 1988) and sea water (Lee *et al.*, 1977) to sandstones (Wildung *et al.,* 2000) and human clinical isolates (Dhawan & Argwal, 1998). What makes *Shewanella* spp unique is its ability to reduce a wide range of electron acceptors, from highly electropositive compounds (e.g. oxygen) to very electronegative acceptors (e.g. sulfite). This behaviour is supported by a complex respiratory pathway, as revealed by genome analysis. Using amino acid sequences of known enzymes, over 40 respiratory proteins were identified in the *Shewanella* MR-1 genome. These include:

- proteins which have been thought of as being absent from *Shewanella* spp: pyruvate formate-lyase and formate-hydrogen-lyase, both enzymes involved in fermentation;
- proteins that have been studied, without the determination of the DNA sequence: TMAO reductase, nitrate and nitrite reductase;
- enzymes which were presumed to be present, but no confirmational studies had been carried out: cytochrome *c* oxidase, cytochrome *c* peroxidase and the bc_1 complex;
- proteins identified in other strains and found in *Shewanella* MR-1 as well: Shp208 (oxygen binding protein), split-Soret diheme, cytochromes c_4;
- proteins already identified and studied both genetically and biochemically in *Shewanella* MR-1: fumarate reductase, CymA (putative electron shuttle), OmcA, MtrC, MtrA and MtrB (decaheme cytochromes involved in iron and manganese respiration), cytochrome c_3 (putative electron shuttle to iron(III) reductase), or similar

to these: Fcc2, Fcc54, Fcc56, Fcc63, Fcc342 and Frd (all fumarate reductase-like proteins), TorC, Cyc129 and others;

- proteins with no homology with known respiratory enzymes.

An interesting group of proteins identified by genome analysis, belongs to the **fumarate reductase** family. Fumarate reductase (Fcc_3) catalyses the reduction of fumarate to succinate. Unlike the *E. coli* enzyme which consists of four subunits and catalyses a reversible reaction, the *Shewanella* MR-1 fumarate reductase is formed by a single polypeptide chain and contains non-covalently bound FAD, in this family a characteristic feature for an unidirectional enzyme. This protein has been isolated, characterised and the crystal structure determined (Leys *et al.,* 1999). By inactivating the corresponding gene (*fccA*) by transposon mutagenesis (Myers & Myers, 1997a) it was shown that the ability of *Shewanella* MR-1 to respire on fumarate was abolished. Apart from this protein, other fumarate reductase-like proteins were identified. One of these, Fcc_2, is similar to iron-fumarate (Ifc_3) reductase identified in another strain, *S. frigidimarina* NCIMB400. Ifc_3, although induced only during iron(III) respiration, is not essential for Fe(III) reduction, possesses a unidirectional fumarate reductase activity and exhibits amino acid sequence and behaviour of the redox centres similar to Fcc_3 (Dobbin et al., 1999).

Another fumarate reductase resembles the enzyme isolated from *W. succinogenes*. FrdA is the catalytic subunit which binds FAD covalently; FrdB contains three iron-sulfur clusters: [2Fe-2S], [3Fe-4S] and [4Fe-4S] and FrdC is a membrane-

bound *b*-type cytochrome. In *W. succinogenes*, the second FrdC subunit ($FrdC_2$) is a diheme cytochrome *b* not required for fumarate respiration (Simon *et al.*, 1998).

Another set of fumarate reductases resembles Fcc_3 (Fcc54, Fcc56, Fcc63 and Fcc342). While Fcc_3 is encoded by one gene (*fccA*), these other proteins are each encoded by a cluster of two or three genes, one for a flavoprotein (*fccA*), one for a tetraheme *c*-type cytochrome (*fccB*) and in Fcc56 and Fcc63, a third reading frame encodes a histidine-ammonia lyase-like protein (*hal*). At the level of amino acid sequences, the flavoprotein and tetraheme cytochrome together are similar to Fcc_3 (around 40 %), but the organisation is different: in Fcc_3 a single polypeptide contains a cytochrome domain situated toward the N-terminal end and the flavin domain at the C-terminal end. In Fcc54 and Fcc342 the two domains are two individual proteins, with the flavoprotein first, followed by the tetraheme *c*-type cytochrome. The Fcc56 operon additionally encodes a Hal protein, after the tetraheme cytochrome, whereas in Fcc63 this is situated between the flavoprotein and the cytochrome. The biological significance of these proteins is not known, but modelling studies showed that although the secondary structure expressed by these proteins is very similar to that of Fcc_3 (~70 %), some of the amino acids involved in the substrate binding and highly conserved in the fumarate reductase family, are not present (Bilsland, unpublished results). The active site may accommodate monocarboxylate substrates like acrylates and cinnamic acid rather than a dicarboxylate, like fumarate. This organisation is similar to methacrylate reductase from *G. sulfurreducens* which presents a flavoprotein and a *c*-type cytochrome organised into an operon. One of these proteins (**<u>FccA56</u>**) was studied in more detail. The *fccA56* gene was isolated by PCR using genomic DNA as a template and the protein

expressed in *Shewanella* MR-1 (using the pMMB503EH expression vector) and *E. coli* (using the pT7-7 vector). During purification, FccA56 expressed in *Shewanella* MR-1 co-purified with other proteins which made the process difficult. The overexpression in *E. coli* was superior to that observed in *Shewanella* and after DEAE-Sephacel and hydroxyapatite columns, the protein was ~ 95 % pure. A UV-visible absorption spectrum confirmed that the ~ 52kDa isolated protein contained flavin. Once isolated, FccA56 was tested for its ability to reduce different substrates like acrylates, cinnamic acid, caffeic acid and others. With the exception of fumarate, FccA56 showed no activity with any other potential substrate. The measured Km was 220 μM and the k_{cat} was 0.15 s^{-1}. These values indicate that fumarate is not a true substrate for FccA56. The mid-point potential of the flavin cofactor was determined following the absorbance changes at 450 nm during titration, using sodium dithionite as a reductant. The values were plotted and the curve was fitted to a two electron transfer Nernst equation. The measured mid-point potentials were -212 and -190 mV. The presence of a flavin semiquinone could not be detected spectrophotometrically.

After purification, the protein was found to contain only 7 % of its flavin and this may be due to the location of the active site at the surface of the protein or incorrect folding due to the absence of the cytochrome and possibly Hal subunit as well. For this reason, *fccA56* + *fccB56* and *fccA56* + *fccB56* + *hal* genes were cloned and protein expression and purification studies need to be carried out in order to determine the most stable and active form of the complex. The *fccA56* gene was successfully inactivated by disruption with a kanamycin resistance cassette. The resulting construct was cloned into

a suicide plasmid (pEP185.2). The *ΔfccA56* strain was grown on minimal media supplemented with lactate and different electron acceptors, but no significant phenotype differences between the deleted strain and the wild-type were noted.

In conclusion, although similar to the flavin domain of Fcc_3, FccA56 is not a fumarate reductase and with the exception of fumarate, none of the tested compounds acted as a substrate. The Fcc56 complex might be an oxido-reductase but non-essential for anaerobic respiration.

Another remarkable feature of *Shewanella* spp which has not been found yet in any other Gram-negative bacterium is the presence of ***c*-type cytochromes in the outer membrane** (Myers & Myers, 1992a). It is thought that this location would facilitate the contact between the cell and the insoluble iron(III) and manganese(IV) oxides which *Shewanella* can metabolise anaerobically. Four of these outer membrane proteins have already been studied (MtrC, MtrA, MtrB and OmcA). Clustered together with these proteins are three more decahemes (MtrF, MtrD and MtrE), presumably with the same cellular location and similar functions. In different locations on the genome two more decahemes have been identified: Cyc18 and Cyc129. **Cyc129** was chosen for further studies. The encoding gene was amplified from the genome, with the lipoprotein signal sequence, and cloned into pMMB503EH for overexpression in *Shewanella* MR-1. Preliminary purification trials showed that after sonication part of the protein is found in the soluble fraction, which indicates a loose association with the membrane. Previous studies indicated that all decahemes studied so far in *Shewanella* MR-1 are involved in

iron and manganese respiration and a similar role was assumed for Cyc129. Gene inactivation was carried out by kanamycin resistance cassette insertion and cloning of the altered gene into the pEP185.2 suicide vector. The ability of the new strain to grow on minimal media supplemented with lactate and different electron acceptors, including iron(III) and manganese(IV) oxides, was then assessed. No differences were noted between the wild-type and the *cyc129* knockout strain. However *Shewanella* spp have been reported to be involved in the conversion of soluble, toxic compounds like uranium(VI), chromium(VI) and probably others, to insoluble and less bioavailable products. Cyc129 could be involved in one of these pathways, either on its own or associated with other proteins in a complex (like uranium(VI) reductase which is a multicomponent enzyme system).

Among the proteins that contained heme binding sites but have no homology with known respiratory enzymes is **Cyc202**. Cyc202 is a soluble protein, and is only the second heptaheme identified so far in any bacterium. Disruption of the encoding sequence by insertion of a kanamycin cassette did not alter the phenotype, compared to the wild-type, except for manganese(IV) oxide reduction. The deleted strain metabolised Mn(IV) slower than the wild-type and this suggested that Cyc202 could be involved in electron transport to the membrane bound iron-manganese reductases.

Other proteins of the anaerobic pathway identified by genome analysis were TMAO reductase, nitrate reductase and nitrite reductase.

The **TMAO reductase** identified is encoded by the *torCAD* operon. TorA is the catalytic subunit and contains a molybdenum binding site. TorC is a membrane-bound pentaheme *c*-type cytochrome involved in electron transfer to TorA and TorD encodes a putative cytoplasmic TorA chaperone.

From the nitrate ammonification pathway, two enzymes have been identified: nitrate reductase and formate dependent nitrite reductase. The periplasmic **nitrate reductase** is encoded by the *napDAGHB* operon and exhibits an organisation similar to that found in *E. coli*. NapA is the catalytic subunit with a molybdopterin binding site; NapB is a diheme *c*-type cytochrome. NapC is replaced by the tetraheme c-type cytochrome CymA. Transposon inactivation of the encoding gene results in an inability of the bacterium to grow on fumarate, nitrate, Fe(III) and Mn(IV), but it is not required for TMAO respiration. The function of the other Nap proteins is unknown. **Nitrite reductase** is represented only by the NrfA protein, which contains the unusual CxxCK heme binding site. Although it has been reported that *Shewanella* MR-1 produces Nar and Nir enzymes, involved in denitrification (Krause & Nealson, 1997), no such proteins were identified when the genome was searched using known amino acid sequences.

From the aerobic respiration cytochrome oxidase, cytochrome reductase and cytochrome peroxidase were identified. The **cytochrome *c* oxidase** identified in *Shewanella* MR-1 and encoded by the *ccoNOPQ* operon is a cbb_3-type oxidase, similar to those isolated from *V. cholerae* and *R. sphaeroides*.

Other complexes of the aerobic respiration found were **cytochrome *c* peroxidase** (catalyses the reduction of hydrogen peroxide to water) and a **cytochrome bc_1 complex** which comprises cytochrome *b*, cytochrome c_1 and an iron-sulfur protein, as in *V. cholerae* and *P. aeruginosa*.

Another protein which is probably part of the aerobic respiratory pathway and was isolated first from the photosynthetic bacterium *R. sphaeroides* is **Shp208**. This protein has an unknown biological significance, but it was identified as a protein that transiently binds oxygen.

Conclusion

This study provides a useful tool for gene inactivation in *Shewanella* MR-1. The existing literature is confusing and the results unreliable. By analysing three different origins of replications we conclude that plasmids that contain the R6K origin are most suitable. If a marked inactivation is required, the antibiotic resistance cassette needs to be carefully designed, as extra DNA sequences (like inactivated transposon genes) could influence the recombination between the disrupted gene and the chromosomal copy.

Genome analysis revealed the presence of over 40 *c*-type cytochromes and other respiratory proteins and only three were studied in more details. Cyc129 is a membrane-bound decaheme *c*-type cytochrome. Although all the decahemes studied so far in *Shewanella* MR-1 are involved in Fe(III) and Mn(IV) oxides reduction, this was not the case for Cyc129, as proved by gene inactivation.

FccA56 is a flavoprotein relatively unstable during purification, as a flavin content, and exhibits low activity with fumarate in a methylviologen dependent reduction. None of the other substrates predicted by molecular modelling to bind at the active site interacted with the protein. If the analogy with fumarate reductase is true, then the active site would be exposed and a cytochrome would be required to close the active site in order for the reaction to occur. In the same cluster with *fccA56* are the genes that encode for a tetraheme *c*-type cytochrome (*fccB56*) and histidine-ammonia lyase-like protein (*hal*). The characterisation of FccA56+FccB56 and FccA56+FccB56+Hal56 will be necessary to determine the most stable and catalytically active for of this complex.

Abu GO, Weiner R, Colwell RR (1994) Glucose metabolism and polysaccharide accumulation in the marine bacterium *Shewanella colwelliana. World J. Microbiol. & Biotechnol.,* **10**, 543-46

Alexeeva S, de Kort B, Sawers G, Hellingwerf KJ, de Mattos MJ (2000) Effects of limited aeration and of the ArcAB system on intermediary pyruvate catabolism in *Escherichia coli. J Bacteriol.* **182(17):**4934-40

Alm RA, Trust TJ (1999) Analysis of the genetic diversity of *Helicobacter pylori*: the tale of two genomes. *J Mol Med.* **77(12):**834-46

Ambler RP (1991) Sequence variability in bacterial cytochromes *c*. *Biochim Biophys Acta*. **1058(1):**42-7

Ambler RP, Daniel M, Melis K, Stout CD (1984). The amino acid sequence of the dihaem cytochrome c_4 from the bacterium *Azotobacter vinelandii. Biochem J.* **22(1):**217-27

Andrews SC, Shipley D, Keen JN, Findlay JB, Harrison PM, Guest JR (1992). The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP+ reductases. *FEBS Lett.* **302(3):**247-52

Ansaldi M, Bordi C, Lepelletier M, Mejean V (1999). TorC apocytochrome negatively autoregulates the trimethylamine N-oxide (TMAO) reductase operon in *Escherichia coli. Mol Microbiol.* **33(2):**284-95

Arikawa Y, Enomoto K, Muratsubaki H, Okazaki M (1998) Soluble fumarate reductase isoenzymes from *Saccharomyces cerevisiae* are required for anaerobic growth. *FEMS Microbiol Lett.* **165(1):**111-6

Arslan E, Schulz H, Zufferey R, Kunzler P, Thony-Meyer L (1998) Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the cbb_3 oxidase in *Escherichia coli. Biochem Biophys Res Commun.* **251(3):**744-7

Aubert C, Lojou E, Bianco P, Rousset M, Durand MC, Bruschi M, Dolla A. (1998) The *Desulfuromonas acetoxidans* triheme cytochrome c_7 produced in *Desulfovibrio desulfuricans* retains its metal reductase activity. *Appl Environ Microbiol.* **64(4):**1308-12

Barrett EL, Kwan HS. (1985). Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol.* **39**:131-49

Bates DM, Popescu CV, Khoroshilova N, Vogt K, Beinert H, Munck E, Kiley PJ (2000) Substitution of leucine 28 with histidine in the *Escherichia coli* transcription factor FNR results in increased stability of the [4Fe-4S](2+) cluster to oxygen. *J Biol Chem.* **275(9):**6234-40

Bates DM, Lazazzera BA, Kiley PJ (1995). Characterization of FNR* mutant proteins indicates two distinct mechanisms for altering oxygen regulation of the *Escherichia coli* transcription factor FNR. *J Bacteriol.* **177(14):**3972-8

Beinert H (1990). Recent developments in the field of iron-sulfur proteins. *FASEB J.* **4(8):**2483-91

Beliaev A, Saffarini D, McLaughlin J, Hunnicutt D.(2001) MtrC, an outer membrane decahaem *c* cytochrome required for metal reduction in *Shewanella putrefaciens* MR-1. *Mol Microbiol.* 39(3):722-30

Beliaev AS, Saffarini DA. (1998) *Shewanella putrefaciens mtrB* encodes an outer membrane protein required for Fe(III) and Mn(IV) reduction. *J Bacteriol.* **180(23):**6292-7

Bell LC, Page MD, Berks BC, Richardson DJ, Ferguson SJ (1993). Insertion of transposon Tn5 into a structural gene of the membrane-bound nitrate reductase of *Thiosphaera pantotropha* results in anaerobic overexpression of periplasmic nitrate reductase activity. *J Gen Microbiol.* **139**:3205-14

Bennett B, Berks BC, Ferguson SJ, Thomson AJ, Richardson DJ (1994). Mo(V) electron paramagnetic resonance signals from the periplasmic nitrate reductase of *Thiosphaera pantotropha. Eur J Biochem.* **226(3):**789-98

Berg DE, Berg CM (1983) The transposable element Tn5. *BioTechnology,* **1**, 417-21

Bergmann DJ, Zahn JA, DiSpirito AA (1999). High-molecular-mass multi-*c*-heme cytochromes from *Methylococcus capsulatus* bath. *J Bacteriol.* **181(3):**991-7

Berks BC, Sargent F, Palmer T (2000) The Tat protein export pathway. *Mol Microbiol.* **35(2):**260-74

Berks BC (1996). A common export pathway for proteins binding complex redox cofactors? *Mol Microbiol.* **22(3):**393-404.

Berks BC, Page MD, Richardson DJ, Reilly A, Cavill A, Outen F, Ferguson SJ (1995). Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron-carrying arm of a redox loop. *Mol Microbiol.* **15(2):**319-31

Berry EA, Trumpower BL (1985) Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome bc_1 and cytochrome c-aa_3 complexes. *J Biol Chem.* **260(4):**2458-67

Blasco F, Iobbi C, Ratouchniak J, Bonnefoy V, Chippaux M (1990). Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and

comparison with that encoded by the *narGHJI* operon. *Mol Gen Genet.* **222(1):**104-11

Blattner FR, Burland V, Plunkett G, Sofia HJ, Daniels DL (1993) Analysis of the *Escherichia coli* genome. IV. DNA sequence of the region from 89.2 to 92.8 minutes. *Nucleic Acids Res.* **21(23):**5408-17

Blaut M, Whittaker K, Valdovinos A, Ackrell BA, Gunsalus RP, Cecchini G. (1989) Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. *J Biol Chem.* **264(23):**13599-604

Bogachev AV, Murtazina RA, Skulachev VP (1993). Cytochrome *d* induction in *Escherichia coli* growing under unfavorable conditions. *FEBS Lett.* **336(1):**75-8

Boyington JC, Gladyshev VN, Khangulov SV, Stadtman TC, Sun PD (1997) Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe4S4 cluster. *Science.* 1997 **275(5304):**1305-8

Bratina BJ, Stevenson BS, Green WJ, Schmidt TM. (1998) Manganese reduction by microbes from oxic regions of the lake vanda (Antarctica) water column *Appl Environ Microbiol.* **64(10):**3791-7

Briggs WR, Christie JM, Knieb E, Solomon M (1999) in Flavins and Flavopoteins, p299

Burnes BS, Mulberry MJ, DiChristina TJ (1998) Design and Application of Two Rapid Screening Techniques for Isolation of Mn(IV) Reduction-Deficient Mutants of *Shewanella putrefaciens. Appl Environ Microbiol.* **64(7):**2716-20

Capaldi RA (1990) Structure and function of cytochrome *c* oxidase. *Annu Rev Biochem.* **59**:569-96

Chang AC, Cohen SN (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol.* **134(3):**1141-56

Chaudhry GR, Chaiken IM, MacGregor CH (1983) An activity from *Escherichia coli* membranes responsible for the modification of nitrate reductase to its precursor form. *J Biol Chem.* **258(9):**5828-33

Christensen HE (1994) Cloning and characterisation of the gene encoding cytochrome c_4 from *Pseudomonas stutzeri. Gene.* **144(1):**139-40

Clarke GJ, Ward FB (1988) Purification and properties of trimethylamine-N-oxide from *Shewanella* sp NCIMB400. *J. Gen. Microbiol* **134(2),** 379-386

Cotter PA, Darie S, Gunsalus RP (1992). The effect of iron limitation on expression of the aerobic and anaerobic electron transport pathway genes in *Escherichia coli*. *FEMS Microbiol Lett.* **79(1-3):**227-32

Cotter PA, Gunsalus RP (1992). Contribution of the *fnr* and *arcA* gene products in coordinate regulation of cytochrome *o* and *d* oxidase (*cyoABCDE and cydAB*) genes in *Escherichia coli*. *FEMS Microbiol Lett.***70(1):**31-6

Czjzek M, Dos Santos JP, Pommier J, Giordano G, Mejean V, Haser R (1998). Crystal structure of oxidized trimethylamine N-oxide reductase from *Shewanella massilia* at 2.5 A resolution. *J Mol Biol.***284(2):**435-47

Dagley S (1987) Lessons from biodegradation. *Annu Rev Microbiol.* **41:1**-23

Darwin AJ, Ziegelhoffer EC, Kiley PJ, Stewart V(1998). Fnr, NarP, and NarL regulation of *Escherichia coli* K-12 *napF* (periplasmic nitrate reductase) operon transcription in vitro. *J Bacteriol.* **180(16):**4192-8

Darwin A, Hussain H, Griffiths L, Grove J, Sambongi Y, Busby S, Cole J (1993) Regulation and sequence of the structural gene for cytochrome c_{552} from *Escherichia coli*: not a hexahaem but a 50 kDa tetrahaem nitrite reductase. *Mol Microbiol.* **9(6):**1255-65

Devreese B, Costa C, Demol H, Papaefthymiou V, Moura I, Moura JJ, Van Beeumen J (1997) The primary structure of the split-Soret cytochrome *c* from *Desulfovibrio desulfuricans* ATCC 27774 reveals an unusual type of diheme cytochrome *c*. *Eur J Biochem.* **248(2):**445-51

Dhawan B, Chaudhry R, Mishra BM, Agarwal R (1998) Isolation of *Shewanella putrefaciens* from a rheumatic heart disease patient with infective endocarditis. *J Clin Microbiol.* **36(8):**2394

DiChristina TJ, DeLong EF (1994). Isolation of anaerobic respiratory mutants of *Shewannella putrefaciens* and genetic analysis of mutants deficient in anaerobic growth on Fe3+. *J Bacteriol.* **176(5):**1468-74

Dobbin PS, Butt JN, Powell AK, Reid GA, Richardson DJ (1999) Characterization of a flavocytochrome that is induced during the anaerobic respiration of Fe3+ by *Shewanella frigidimarina* NCIMB400. *Biochem J.* **342** :439-48

Doherty MK, Pealing SL, Miles CS, Moysey R, Taylor P, Walkinshaw MD, Reid GA, Chapman SK (2000) Identification of the active site acid/base catalyst in a bacterial fumarate reductase: a kinetic and crystallographic study. *Biochemistry.* **39(35):**10695-701

Dominguez H, Vogel BF, Gram L, Hoffmann S, Schaebel S (1996) *Shewanella alga* bacteremia in two patients with lower leg ulcers. *Clin Infect Dis.* **22(6):**1036-9

Dos Santos JP, Iobbi-Nivol C, Couillault C, Giordano G, Mejean V (1998). Molecular analysis of the trimethylamine N-oxide (TMAO) reductase respiratory system from a *Shewanella species. J Mol Biol.* **284(2):**421-33

Dubourdieu M, DeMoss JA (1992). The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli. J Bacteriol.* **174(3):**867-72

Easter, MC,DM Gibson and FB Ward (1983). The induction and location of trimethyl-amine N-oxide reductase in *Alteromonas* sp. NCMB 400. *J. Gen. Microbiol.* **129**, 3689-3696.

Einsle O, Messerschmidt A, Stach P, Bourenkov GP, Bartunik HD, Huber R, Kroneck PM (1999) Structure of cytochrome *c* nitrite reductase. *Nature.* **400(6743)**:476-80

Field SJ, Dobbin PS, Cheesman MR, Watmough NJ, Thomson AJ, Richardson DJ (2000) Purification and magneto-optical spectroscopic characterization of cytoplasmic membrane and outer membrane multiheme *c*-type cytochromes from *Shewanella frigidimarina* NCIMB400. *J Biol Chem.* **275(12):**8515-22

Fu HA, Iuchi S, Lin EC (1991) The requirement of ArcA and Fnr for peak expression of the *cyd* operon in *Escherichia* coli under microaerobic conditions. *Mol Gen Genet.* **226(1-2):**209-13

Galimand M, Gamper M, Zimmermann A, Haas D (1991). Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa. J Bacteriol.* **173(5):**1598-606

Garcia-Horsman JA, Berry E, Shapleigh JP, Alben JO, Gennis RB (1994). A novel cytochrome c oxidase from *Rhodobacter sphaeroides* that lacks CuA. *Biochemistry.* **33(10):**3113-9

Gaspard S, Vazquez F, Holliger C (1998) Localization and solubilization of the Iron(III) reductase of *Geobacter sulfurreducens Appl Environ Microbiol.* **64(9):**3188-94

Gay P, Le Coq D, Steinmetz M, Ferrari E, Hoch JA (1983) Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *Escherichia coli. J Bacteriol.* **153(3):**1424-31

Ge Z, Feng Y, Dangler CA, Xu S, Taylor NS, Fox JG (2000) Fumarate reductase is essential for *Helicobacter pylori* colonization of the mouse stomach. *Microb Pathog.* **29(5):**279-87

Ge Z, Jiang Q, Kalisiak MS, Taylor DE (1997) Cloning and functional characterization of *Helicobacter pylori* fumarate reductase operon comprising three structural genes coding for subunits C, A and B. *Gene* **204(1-2):**227-34

Ghisla S, Massey V. (1989). Mechanisms of flavoprotein-catalyzed reactions. *Eur J Biochem.* **181(1):**1-17

Ghisla S, Massey V (1986). New flavins for old: artificial flavins as active site probes of flavoproteins. *Biochem J.***239(1):**1-12

Giudici-Orticoni MT, Leroy G, Nitschke W, Bruschi M (2000) Characterization of a new dihemic $c_{(4)}$-type cytochrome isolated from *Thiobacillus ferrooxidans. Biochemistry.* **39(24):**7205-11

Gray GO, Gaul DF, Knaff DB.(1983) Partial purification and characterization of two soluble *c*-type cytochromes from *Chromatium vinosum. Arch Biochem Biophys.* **222(1):**78-86

Grove J, Tanapongpipat S, Thomas G, Griffiths L, Crooke H, Cole J (1996) *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. *Mol Microbiol.* **19(3):**467-81

Gordon EH, Pike AD, Hill AE, Cuthbertson PM, Chapman SK, Reid GA (2000) Identification and characterization of a novel cytochrome c_3 from *Shewanella frigidimarina* that is involved in Fe(III) respiration. *Biochem J.* **349(Pt 1):**153-8

Gordon EH, Pealing SL, Chapman SK, Ward FB, Reid GA (1998). Physiological function and regulation of flavocytochrome *c*3, the soluble fumarate reductase from *Shewanella putrefaciens* NCIMB 400. *Microbiology.* **144 (4):**937-45

Gray GO, Gaul DF, Knaff DB (1983) Partial purification and characterization of two soluble *c*-type cytochromes from *Chromatium vinosum. Arch Biochem Biophys.* **222(1):**78-86

Green J, Bennett B, Jordan P, Ralph ET, Thomson AJ, Guest JR (1996). Reconstitution of the [4Fe-4S] cluster in FNR and demonstration of the aerobic-anaerobic transcription switch in vitro. *Biochem J.* **316 (3):**887-92

Green J, Guest JR (1993a). Activation of FNR-dependent transcription by iron: an in vitro switch for FNR. *FEMS Microbiol Lett.* **113(2):**219-22

Green J, Sharrocks AD, Green B, Geisow M, Guest JR (1993b). Properties of FNR proteins substituted at each of the five cysteine residues. *Mol Microbiol.* **8(1):**61-8

Green J, Guest JR (1993c). A role for iron in transcriptional activation by FNR. *FEBS Lett.* **329(1-2):**55-8

Grove J, Tanapongpipat S, Thomas G, Griffiths L, Crooke H, Cole J (1996). *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. *Mol Microbiol.* **19(3):**467-81

Guigliarelli B, Magalon A, Asso M, Bertrand P, Frixon C, Giordano G, Blasco F (1996) Complete coordination of the four Fe-S centers of the beta subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of site-directed mutants lacking the highest or lowest potential [4Fe-4S] clusters. *Biochemistry.* **35(15):**4828-36

Hershfield V, Boyer HW, Yanofsky C, Lovett MA, Helinski DR (1974). Plasmid ColEl as a molecular vehicle for cloning and amplification of DNA. *Proc Natl Acad Sci U S A.* **71(9):**3455-9

Hill JJ, Alben JO, Gennis RB (1993). Spectroscopic evidence for a heme-heme binuclear center in the cytochrome bd ubiquinol oxidase from *Escherichia coli. Proc Natl Acad Sci U S A.* **90(12):**5863-7

Hill S, Viollet S, Smith AT, Anthony C (1990). Roles for enteric *d*-type cytochrome oxidase in N_2 fixation and microaerobiosis. *J Bacteriol.* **172(4):**2071-8

Hochkoeppler A, Jenney FE, Lang SE, Zannoni D, Daldal F (1995). Membrane-associated cytochrome c_y of *Rhodobacter capsulatus* is an electron carrier from the cytochrome bc_1 complex to the cytochrome *c* oxidase during respiration. *J Bacteriol.* **177(3):**608-13

Hoffmann T, Frankenberg N, Marino M, Jahn D (1998). Ammonification in *Bacillus subtilis* utilizing dissimilatory nitrite reductase is dependent on *resDE. J Bacteriol.* **180(1):**186-9

Hu W, Van Driessche G, Devreese B, Goodhew CF, McGinnity DF, Saunders N, Fulop V, Pettigrew GW, Van Beeumen JJ (1997) Structural characterization of *Paracoccus denitrificans* cytochrome *c* peroxidase nd assignment of the low and high potential heme sites. *Biochemistry.* **36(26):**7958-66

Ioannidis N, Cooper CE, Poole RK (1992). Spectroscopic studies on an oxygen-binding haemoglobin-like flavohaemoprotein from *Escherichia coli. Biochem J.* **288 (2)**:649-55

Iuchi S (1993). Phosphorylation/dephosphorylation of the receiver module at the conserved aspartate residue controls transphosphorylation activity of histidine kinase in sensor protein ArcB of *Escherichia coli. J Biol Chem.* **268(32):**23972-80

Iuchi S, Lin EC (1992). Mutational analysis of signal transduction by ArcB, a membrane sensor protein responsible for anaerobic repression of operons involved in the central aerobic pathways in *Escherichia coli. J Bacteriol.* **174(12):**3972-80

Iuchi S, Chepuri V, Fu HA, Gennis RB, Lin EC (1990a). Requirement for terminal cytochromes in generation of the aerobic signal for the *arc* regulatory system in *Escherichia coli*: study utilizing deletions and *lac* fusions of *cyo* and *cyd*. *J Bacteriol*. **172(10):**6020-5

Iuchi S, Matsuda Z, Fujiwara T, Lin EC (1990b). The *arcB* gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the *arc* modulon. *Mol Microbiol.* **4(5):**715-27

Iuchi S, Lin EC (1988). *arcA* (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. *Proc Natl Acad Sci U S A.* **85(6):**1888-92

Iverson TM, Luna-Chavez C, Cecchini G, Rees DC (1999) Structure of the *Escherichia coli* fumarate reductase respiratory complex. *Science.* **284(5422):**1961-6

Jennings MP, Beacham IR (1993). Co-dependent positive regulation of the *ansB* promoter of *Escherichia coli* by CRP and the FNR protein: a molecular analysis. *Mol Microbiol.* **9(1):**155-64

Johnson MK, Kowal AT, Morningstar JE, Oliver ME, Whittaker K, Gunsalus RP, Ackrell BA, Cecchini G (1988) Subunit location of the iron-sulfur clusters in fumarate reductase from *Escherichia coli. J Biol Chem.* **263(29):**14732-8

Jorns MS, Wang B, Jordan SP (1987). DNA repair catalyzed by *Escherichia coli* DNA photolyase containing only reduced flavin: elimination of the enzyme's second chromophore by reduction with sodium borohydride. *Biochemistry*. **26(21):**6810-6

Jourlin C, Bengrine A, Chippaux M, Mejean V (1996). An unorthodox sensor protein (TorS) mediates the induction of the *tor* structural genes in response to trimethylamine N-oxide in *Escherichia coli. Mol Microbiol*. **20(6):**1297-306

Khashe S, Janda JM (1998) Biochemical and pathogenic properties of *Shewanella alga* and *Shewanella putrefaciens*. *J Clin Microbiol.* **36(3):**783-7

Khoroshilova N, Popescu C, Munck E, Beinert H, Kiley PJ (1997). Iron-sulfur cluster disassembly in the FNR protein of *Escherichia coli* by O_2: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. *Proc Natl Acad Sci U S A.* **94(12):**6087-92.

Khoroshilova N, Beinert H, Kiley PJ (1995). Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding. *Proc Natl Acad Sci U S A.* **92(7):**2499-503.

Kinder SA, Badger JL, Bryant GO, Pepe JC, Miller VL (1993). Cloning of the YenI restriction endonuclease and methyltransferase from *Yersinia enterocolitica*

serotype O8 and construction of a transformable R-M+ mutant. *Gene.* 1993 **136(1-2)**:271-5

Klarskov K, Van Driessche G, Backers K, Dumortier C, Meyer TE, Tollin G, Cusanovich MA, Van Beeumen JJ (1998) Ligand binding and covalent structure of an oxygen-binding heme protein from *Rhodobacter sphaeroides*, a representative of a new structural family of *c*-type cytochromes. *Biochemistry.* **37(17):**5995-6002

Klinger A, Schirawski J, Glaser P, Unden G (1998). The *fnr* gene of *Bacillus licheniformis* and the cysteine ligands of the C-terminal FeS cluster. *J Bacteriol.* **180(13):**3483-5.

Kolter R, Inuzuka M, Helinski DR (1978). Trans-complementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. *Cell.* **15(4):**1199-208

Krause B, Nealson K (1997) Physiology and enzymology involved in denitrification by *Shewanella putrefaciens. Appl. Environ. Microbiol.,* **63,** 2613-18

Kuo CF, McRee DE, Fisher CL, O'Handley SF, Cunningham RP, Tainer JA (1992). Atomic structure of the DNA repair [4Fe-4S] enzyme endonuclease III. *Science.* **258(5081):**434-40

Kurowski B, Ludwig B (1987). The genes of the *Paracoccus denitrificans* bc_1 complex. Nucleotide sequence and homologies between bacterial and mitochondrial subunits. *J Biol Chem.* **262(28):**13805-11

Lancaster CR, Kroger A, Auer M, Michel H (1999) Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 A resolution. *Nature.* **402(6760):**377-85

Lee JV, Bibson DM, Shewan JM (1977) A numerical taxonomic study of some *Pseudomonas* like marine bacteria. *J. Gen. Microbiol.,* **98**, 439-51

Leys D, Tsapin AS, Nealson KH, Meyer TE, Cusanovich MA, Van Beeumen JJ (1999) Structure and mechanism of the flavocytochrome *c* fumarate reductase of *Shewanella putrefaciens* MR-1. *Nat Struct Biol.* **6(12):**1113-7

Lin EC, Iuchi S (1991) Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. *Annu Rev Genet.* **25:**361-87

Liu MC, Costa C, Coutinho IB, Moura JJ, Moura I, Xavier AV, LeGall J (1988) Cytochrome components of nitrate- and sulfate-respiring *Desulfovibrio desulfuricans* ATCC 27774. *J Bacteriol.* **170(12):**5545-51

MacAllister TW, Kelley WL, Miron A, Stenzel TT, Bastia D (1991) Replication of plasmid R6K origin gamma in vitro. Dependence on dual initiator proteins and inhibition by transcription. *J Biol Chem.* **266(24):**16056-62

MacDonell MT, Colwell RR (1985) Phylogeny of *Vibrionaceae*, and recommendation for two new genera, *Listonella* and *Shewanella*. *Syst. Appl. Microbiol.* **6:** 171-182

Malatesta F, Antonini G, Sarti P, Brunori M (1995) Structure and function of a molecular machine: cytochrome *c* oxidase. *Biophys Chem.* **54(1):**1-33

Matias PM, Morais MA, Coelho AV, Meijers R, Gonzales A, Thompson AW, Sieker L, LeGall J, Carrondo MA (1997) A preliminary analysis of the three-dimensional structure of dimeric dihaem spli-Soret cytochrome *c* from *Desulfovibrio desulfuricans* ATCC27774 at 2.5 Å resolution using the MAD phasing method: a novel cytochrome fold with a stacked-heme arrangement. *J. Biol. Inorg. Chem.,* **2**, 507-512

Mejean V, Iobbi-Nivol C, Lepelletier M, Giordano G, Chippaux M, Pascal MC (1994). TMAO anaerobic respiration in *Escherichia coli:* involvement of the *tor* operon. *Mol Microbiol.* **11(6):**1169-79.

Melville SB, Gunsalus RP (1996). Isolation of an oxygen-sensitive FNR protein of *Escherichia coli*: interaction at activator and repressor sites of FNR-controlled genes. *Proc Natl Acad Sci U S A.* **93(3):**1226-31

Michel LO, Sandkvist M, Bagdasarian M (1995) Specificity of the protein secretory apparatus: secretion of the heat-labile enterotoxin B subunit pentamers by different species of gram- bacteria. *Gene.* **152(1):**41-5

Mikoulinskaia O, Akimenko V, Galouchko A, Thauer RK, Hedderich R (1999) Cytochrome *c*-dependent methacrylate reductase from *Geobacter sulfurreducens* AM-1. *Eur J Biochem.* **263(2):**346-52

Miller VL, Mekalanos JJ (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol.* **170(6):**2575-83

Montoya G, te Kaat K, Rodgers S, Nitschke W, Sinning I (1999) The cytochrome bc_1 complex from *Rhodovulum sulfidophilum* is a dimer with six quinones per monomer and an additional 6-kDa component. *Eur J Biochem.* **259(3):**709-18

Mouncey NJ, Kaplan S (1998) Oxygen regulation of the *ccoN* gene encoding a component of the cbb_3 oxidase in *Rhodobacter sphaeroides* 2.4.1T: involvement of the FnrL protein. *J Bacteriol.* **180(8):**2228-31

Murphy MJ, Siegel LM (1973). Siroheme and sirohydrochlorin. The basis for a new type of porphyrin-related prosthetic group common to both assimilatory and dissimilatory sulfite reductases. *J Biol Chem.* **248(19):**6911-9

Myers JM, Myers CR (2001) Role for outer membrane cytochromes OmcA and OmcB of *Shewanella putrefaciens* MR-1 in reduction of manganese dioxide. *Appl Environ Microbiol.* **67(1):**260-9.

Myers CR, Carstens BP, Antholine WE, Myers JM (2000) Chromium(VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J Appl Microbiol.* **88(1):**98-106.

Myers JM, Myers CR (2000) Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *J Bacteriol.* **182(1):**67-75.

Myers JM, Myers CR (1998) Isolation and sequence of *omcA,* a gene encoding a decaheme outer membrane cytochrome *c* of *Shewanella putrefaciens* MR-1, and detection of *omcA* homologs in other strains of *S. putrefaciens. Biochim Biophys Acta.* **1373(1):**237-51.

Myers CR, Myers JM (1997a) Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. *J Bacteriol.* **179(4):**1143-52

Myers CR, Myers JM (1997b). Isolation and characterization of a transposon mutant of *Shewanella putrefaciens* MR-1 deficient in fumarate reductase. *Lett Appl Microbiol.* **25(3):**162-8.

Myers CR, Myers JM (1997c). Replication of plasmids with the *p15A* origin in *Shewanella putrefaciens* MR-1. *Lett Appl Microbiol.* **24(3):**221-5

Myers CR, Myers JM (1992) Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J Bacteriol.* **174(11):**3429-38.

Myers CR, Nealson KH (1990) Respiration-linked proton translocation coupled to anaerobic reduction of manganese(IV) and iron(III) in *Shewanella putrefaciens* MR 1. *J Bacteriol.* **172(11):**6232-8

Myllykallio H, Drepper F, Mathis P, Daldal F (1998). Membrane-anchored cytochrome c_y mediated microsecond time range electron transfer from the cytochrome bc_1 complex to the reaction center in *Rhodobacter capsulatus. Biochemistry* **37(16):**5501-10.

Nakano MM, Zuber P (1998). Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). *Annu Rev Microbiol.* **52**:165-90.

Nakano MM, Dailly YP, Zuber P, Clark DP (1997). Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. *J Bacteriol.* **179(21):**6749-55.

Nakano MM, Zuber P, Glaser P, Danchin A, Hulett FM (1996). Two-component regulatory proteins ResD-ResE are required for transcriptional activation *of fnr* upon oxygen limitation in *Bacillus subtilis. J Bacteriol.* **178(13):**3796-802

Nichols DS, Olley J, Garda H, Brenner RR, McMeekin TA (2000) Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina. Appl Environ Microbiol.* **66(6):**2422-9

Nozue H, Hayashi T, Hashimoto Y, Ezaki T, Hamasaki K, Ohwada K, Terawaki Y (1992) Isolation and characterization of *Shewanella alga* from human clinical specimens and emendation of the description of *S. alga* Simidu et al., 1990, 335. *Int J Syst Bacteriol.* **42(4):**628-34

Obuekwe CO, Westlake DW (1982) Effects of medium composition on cell pigmentation, cytochrome content, and ferric iron reduction in a *Pseudomonas* sp. isolated from crude oil. *Can J Microbiol.* 28(8):989-92

Pealing SL, Black AC, Manson FD, Ward FB, Chapman SK, Reid GA (1992) Sequence of the gene encoding flavocytochrome *c* from *Shewanella putrefaciens*: a tetraheme flavoenzyme that is a soluble fumarate reductase related to the membrane-bound enzymes from other bacteria. *Biochemistry.* **31(48):**12132-40

Pepe JC, Miller VL (1993). *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. *Proc Natl Acad Sci U S A.* **90(14):**6473-7

Pereira MM, Carita JN, Teixeira M (1999) Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: a novel multihemic cytochrome *bc*, a new complex III. *Biochemistry.* **38(4):**1268-75

Pommier J, Mejean V, Giordano G, Iobbi-Nivol C (1998). TorD, a cytoplasmic chaperone that interacts with the unfolded trimethylamine N-oxide reductase enzyme (TorA) in *Escherichia coli. J Biol Chem.* **273(26):**16615-20.

Poole RK, Anjum MF, Membrillo-Hernandez J, Kim SO, Hughes MN, Stewart V (1996). Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. *J Bacteriol.* **178(18):**5487-92.

Potter LC, Cole JA (1999). Essential roles for the products of the *napABCD* genes, but not *napFGH*, in periplasmic nitrate reduction by *Escherichia coli* K-12. *Biochem J.* **344** :69-76

Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev.* **57(1):**50-108

Quandt J, Hynes MF (1993). Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene.* **127(1):**15-21

Rabin RS, Stewart V (1993). Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. *J Bacteriol.* **175(11):**3259-68

Reincke B, Thony-Meyer L, Dannehl C, Odenwald A, Aidim M, Witt H, Ruterjans H, Ludwig B. (1999) Heterologous expression of soluble fragments of cytochrome c_{552} acting as electron donor to the *Paracoccus denitrificans* cytochrome *c* oxidase. *Biochim Biophys Acta.* **1411(1):**114-20

Reyes F, Gavira M, Castillo F, Moreno-Vivian C (1998). Periplasmic nitrate-reducing system of the phototrophic bacterium *Rhodobacter sphaeroides* DSM 158: transcriptional and mutational analysis of the *napKEFDABC* gene cluster. *Biochem J.* **331 (3):**897-904

Robinson KM, Lemire BD (1995) Flavinylation of succinate: ubiquinone oxidoreductase from *Saccharomyces cerevisiae. Methods Enzymol.* **260:**34-51

Roldan MD, Sears HJ, Cheesman MR, Ferguson SJ, Thomson AJ, Berks BC, Richardson DJ (1998) Spectroscopic characterization of a novel multiheme *c*-type cytochrome widely implicated in bacterial electron transport. *J Biol Chem.* **273(44):**28785-90

Rothstein SJ, Jorgensen RA, Yin JC, Yong-di Z, Johnson RC, Reznikoff WS (1981) Genetic organization of Tn5. *Cold Spring Harb Symp Quant Biol.* **45(1):**99-105

Rowe JJ, Ubbink-Kok T, Molenaar D, Konings WN, Driessen AJ (1994). NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration *by Escherichia coli. Mol Microbiol.* **12(4):**579-86.

Saffarini DA, Nealson KH (1993). Sequence and genetic characterization of *etrA*, an *fnr* analog that regulates anaerobic respiration in *Shewanella putrefaciens* MR-1. *J Bacteriol.* **175(24):**7938-44.

Sambasivarao D, Turner RJ, Simala-Grant JL, Shaw G, Hu J, Weiner JH (2000). Multiple roles for the twin arginine leader sequence of dimethyl sulfoxide reductase of *Escherichia coli. J Biol Chem*. **275(29):**22526-31

Samuelsson MO (1985) Dissimilatory nitrate reduction to nitrate, nitrous oxide, and ammonium by *Pseudomonas putrefaciens. Appl Environ Microbiol.* **50(4):**812-5

Samyn B, Van Craenenbroeck K, De Smet L, Vandenberghe I, Pettigrew G, Van Beeumen J (1995) A reinvestigation of the covalent structure of *Pseudomonas aeruginosa* cytochrome *c* peroxidase. *FEBS Lett.* **377(2):**145-9

Sawers G, Suppmann B (1992). Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. *J Bacteriol.* **174(11):**3474-8.

Schroder I, Wolin CD, Cavicchioli R, Gunsalus RP (1994). Phosphorylation and dephosphorylation of the NarQ, NarX, and NarL proteins of the nitrate-dependent two-component regulatory system of *Escherichia coli. J Bacteriol.* **176(16):**4985-92

Schwartz CJ, Djaman O, Imlay JA, Kiley PJ (2000) The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli. Proc Natl Acad Sci U S A.* **97(16):**9009-14.

Schwede TF, Retey J, Schulz GE (1999) Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. *Biochemistry.* **38(17):**5355-61

Shaw DJ, Rice DW, Guest JR (1983). Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli . J Mol Biol.* **166(2):**241-7

Siddiqui RA, Warnecke-Eberz U, Hengsberger A, Schneider B, Kostka S, Friedrich B (1993). Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. *J Bacteriol.* **175(18):**5867-76.

Silvestro A, Pommier J, Giordano G (1988). The inducible trimethylamine-N-oxide reductase of *Escherichia coli* K12: biochemical and immunological studies. *Biochim Biophys Acta.* **954(1):**1-13

Simidu U, Kita-Tsukamoto K, Yasumoto T, Yotsu M (1990) Taxonomy of four marine bacterial strains that produce tetrodotoxin. *Int J Syst Bacteriol.* **40(4):**331-6

Simon J, Gross R, Einsle O, Kroneck PM, Kroger A, Klimmek O (2000) A NapC/NirT-type cytochrome *c* (NrfH) is the mediator between the quinone pool and the cytochrome *c* nitrite reductase of *Wolinella succinogenes. Mol Microbiol.* **35(3):**686-96

Simon J, Gross R, Ringel M, Schmidt E, Kroger A (1998a) Deletion and site-directed mutagenesis of the *Wolinella succinogenes* fumarate reductase operon. *Eur J Biochem.* **251(1-2):**418-26

Simon J, Gross R, Klimmek O, Ringel M, Kroger A (1998) A periplasmic flavoprotein in *Wolinella succinogenes* that resembles the fumarate reductase of *Shewanella putrefaciens. Arch Microbiol.* **169(5):**424-33

Simons RW, Houman F, Kleckner N (1987) Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene.* **53(1):**85-96

Sodergren EJ, DeMoss JA (1988) *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. *J Bacteriol.* **170(4):**1721-9

Spiro S, Guest JR (1990). FNR and its role in oxygen-regulated gene expression in *Escherichia coli. FEMS Microbiol Rev.* **6(4):**399-428.

Spiro S, Guest JR (1988). Inactivation of the FNR protein of *Escherichia coli* by targeted mutagenesis in the N-terminal region. *Mol Microbiol.* **2(6):**701-7.

Spiro S, Guest JR (1987). Regulation and over-expression of the *fnr* gene of *Escherichia coli. J Gen Microbiol.* **133 (12):**3279-88.

Sun G, Sharkova E, Chesnut R, Birkey S, Duggan MF, Sorokin A, Pujic P, Ehrlich SD, Hulett FM (1996). Regulators of aerobic and anaerobic respiration in *Bacillus subtilis. J Bacteriol.* **178(5):**1374-85.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature.* **397(6718):**441-6.

Svensson B, Lubben M, Hederstedt L (1993). *Bacillus subtilis* CtaA and CtaB function in haem A biosynthesis. *Mol Microbiol.* **10(1):**193-201

Taylor P, Pealing SL, Reid GA, Chapman SK, Walkinshaw MD (1999) Structural and mechanistic mapping of a unique fumarate reductase. *Nat Struct Biol.* **6(12):**1108-12

Toledo-Cuevas M, Barquera B, Gennis RB, Wikstrom M, Garcia-Horsman JA (1998) The cbb_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase. *Biochim Biophys Acta.* **1365(3):**421-34

Trumpower BL, Gennis RB (1994). Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. *Annu Rev Biochem.* **63**:675-716

Tsapin AI, Nealson KH, Meyers T, Cusanovich MA, Van Beuumen J, Crosby LD, Feinberg BA, Zhang C (1996) Purification and properties of a low-redox-potential tetraheme cytochrome c_3 from *Shewanella putrefaciens. J Bacteriol.* **178(21):**6386-8

Unden G, Bongaerts J (1997) Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. *Biochim Biophys Acta.* **1320(3):**217-34

Van Beeumen JJ, Demol H, Samyn B, Bartsch RG, Meyer TE, Dolata MM, Cusanovich MA (1991) Covalent structure of the diheme cytochrome subunit and amino-terminal sequence of the flavoprotein subunit of flavocytochrome *c* from *Chromatium vinosum. J Biol Chem.* **266(20):**12921-31

Vandenberghe I, Leys D, Demol H, Van Driessche G, Meyer TE, Cusanovich MA, Van Beeumen J. (1998) The primary structures of the low-redox potential

diheme cytochromes c from the phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodobacter adriaticus* reveal a new structural family of *c*-type cytochromes. *Biochemistry.* **37(38):**13075-81

Van Hellemond JJ, Tielens AG (1994) Expression and functional properties of fumarate reductase. *Biochem J.* **304:**321-31

Van Spanning RJ, De Boer AP, Reijnders WN, Spiro S, Westerhoff HV, Stouthamer AH, Van der Oost J (1995). Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. *FEBS Lett.* **360(2):**151-4

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14(11):4683-90

Wade R, DiChristina TJ (2000) Isolation of U(VI) reduction-deficient mutants of *Shewanella putrefaciens. FEMS Microbiol Lett.* **184(2):**143-8

Wall D, Delaney JM, Fayet O, Lipinska B, Yamamoto T, Georgopoulos C (1992). *arc*-dependent thermal regulation and extragenic suppression of the *Escherichia coli* cytochrome *d* operon. *J Bacteriol.* **174(20):**6554-62

Wildung RE, Gorby YA, Krupka KM, Hess NJ, Li SW, Plymale AE, McKinley JP, Fredrickson JK (2000) Effect of electron donor and solution chemistry on products of dissimilatory reduction of technetium by *Shewanella putrefaciens. Appl Environ Microbiol.* **66(6):**2451-60

Williams R, Bell A, Sims G, Busby S (1991) The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins. *Nucleic Acids Res.* **19(24):**6705-12

Wissenbach U, Ternes D, Unden G (1992). An *Escherichia coli* mutant containing only demethylmenaquinone, but no menaquinone: effects on fumarate, dimethylsulfoxide, trimethylamine N-oxide and nitrate respiration. *Arch Microbiol.* **158(1):**68-73.

Wissenbach U, Kroger A, Unden G (1990). The specific functions of menaquinone and demethylmenaquinone in anaerobic respiration with fumarate, dimethylsulfoxide, trimethylamine N-oxide and nitrate by *Escherichia coli. Arch Microbiol.* **154(1):**60-6

Yamada M, Nakasone K, Tamegai H, Kato C, Usami R, Horikoshi K (2000) Pressure regulation of soluble cytochromes c in a deep-Sea piezophilic bacterium, *Shewanella violacea. J Bacteriol.* **182(10):**2945-52

Yohe S, Fishbain JT, Andrews M (1997). *Shewanella putrefaciens* abscess of the lower extremity. *J Clin Microbiol.* **35(12):**3363

Zimmermann A, Reimmann C, Galimand M, Haas D (1991) Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli. Mol Microbiol.* **5(6):**1483-90