Characterisation of cytochromes c_3 and c_5 from *Shewanella frigidimarina* NCIMB400

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Declaration

I declare that this thesis was composed by myself and the work presented herein is my own other than where referenced to others.

Anne E. Hill

December 1998

For mum and dad

Acknowledgements

Firstly, many thanks to all occupants, both past and present, of the Reid/Chapman laboratories. In particular to Dr Stuart Rivers and Douglas Robertson, for endless cups of tea and chatter, and my supervisors Dr Graeme Reid, Prof. Steve Chapman, and Dr Bruce Ward.

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Abstract

Shewanella frigidimarina NCIMB400 is able to survive in a variety of environments due to its ability to utilise a wide array of electron donors and acceptors for respiration. This flexibility is a result of its capacity to produce a series of highly branched respiratory chains, rich in cytochromes, the specific components of which are expressed in response to the particular electron donors and acceptors available. Several *c*-type cytochromes have been identified in preliminary studies of the respiratory chains of *Shewanella frigidimarina* NCIMB400. In order to extend our understanding of the nature of these respiratory chains, two of the small *c*-type cytochromes, cytochrome c_3 and cytochrome c_5, have been the focus of more comprehensive study.

The structural gene encoding cytochrome c_5 has been cloned and sequenced, along with some surrounding sequence. The inferred amino acid sequence of the cloned gene, *scy*A, corresponds to a mature protein of 78 amino acids with a single haem attachment motif situated toward the N-terminal end of the protein; a methionine residue near the C-terminus serves as the sixth haem ligand. The *scy*A open reading frame contains a 21 amino acid N-terminal extension which is absent in purified cytochrome c_5. This sequence conforms to the format of a typical periplasmic signal sequence.

Two additional open reading frames were identified on analysis of the regions flanking the structural gene, neither of which is functionally related to cytochrome c_5. Northern blot analysis confirmed that *scy*A is transcriptionally isolated.

In order to investigate the physiological role of cytochrome c_5, a null mutant which lacked the gene coding for cytochrome c_5 was constructed. The anaerobic respiratory capacity of the resultant strain was assessed and compared to wild-type. No obvious mutant phenotype was identified.

Gene disruption experiments were also used to characterise cytochrome c_3. Deletion strains lacking the gene coding for cytochrome c_3 (*cct*A) and also strains lacking both cytochrome c_3 and flavocytochrome c_3 were constructed. Comparison of the growth

characteristics of the mutant strains with wild-type suggest the involvement of cytochrome c_3 with respiratory iron (III) reduction. Ferrozine extraction experiments similarly demonstrated a decrease in iron (III) reduction activity by strains lacking the cytochrome c_3 gene. No other phenotype could be found for the Δ*cct*A strain.

In order to facilitate further study of cytochrome c_5, and production of recombinant forms of the protein, an expression system was developed. Cytochrome c_5 was successfully expressed in *Shewanella frigidimarina* NCIMB400 by using the expression vector pMMB503 which is inducible with isopropylthio-β-D-galactoside.

Abbreviations

APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CoA	coenzyme A
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytdine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNTP	mixture of dATP, dCTP, dGTP and dTTP
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
E_m	Midpoint potential
FAD	flavin adenine dinucleotide
GTP	guanosine triphosphate
HQNO	2-heptyl-4-hydroxyquinoline N-oxide
IPTG	isopropyl thiogalactoside
kb	kilobase
kDA	kilo Dalton
LB	Luria-Bertani Broth
MCS	multiple cloning site
MQ	MilliQ™ grade water
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NCIMB	National Collection of Industrial and Marine Bacteria
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
P_i	inorganic phosphate
PIPES	1,4-Piperazine-N,N'-bis[2-ethane sulfonic acid]
RBS	Ribosomal Binding Site
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TMAO	trimethylamine oxide
TMBZ	3,3',5,5'-tetramethylbenzidine
Tris	Tris hydroxy methyl aminoethane
tRNA	transfer RNA
UQ	ubiquinone
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Δ	deletion

Nucleic acid sequence abbreviations

A	adenine	**Y**	C or T
C	cytosine	**R**	A or G
G	guanine	**N**	T or C orG or A
T	thymine	**W**	A or T
U	uracil	**S**	C or G

Protein abbreviations

Amino acid	**Three letter abbreviation**	**One letter symbol**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

List of contents

List of figures

List of tables

Chapter 1

Introduction

Inspired by the sheer abundance and diversity of prokaryotic cells on the face of the earth, research into the means by which such relatively simple organisms survive and reproduce has long drawn attention. Bacteria have evolved to thrive in almost all natural environments regardless of the nature of available sources of carbon, energy, and reducing power, or pH, oxidation-reduction potential and the availability of electron acceptors. The black magic or vitalism by which microorganisms thrive in such a remarkable range of environments is only now, in the advent of powerful molecular genetic techniques, beginning to be fully elucidated. Members of the genus *Shewanella* stand out among respiratory organisms as facultative anaerobes with exceptional anaerobic respiratory plasticity, prevalent in a variety of different environments, many of which are suboxic or anoxic (Morris *et al.*, 1990; Wilkinson and Caudwell, 1980). As such, *Shewanella* provide an interesting subject for studies of the metabolic capabilities of prokaryotic microorganisms.

1.1 Microbiology of *Shewanella*

Shewanella are Gram-negative, rod-like, motile, facultative anaerobes. These bacteria are members of the gamma three subgroup of proteobacteria, closely related to the enteric bacteria, such as *Escherichia coli* (Gauthier *et al.*, 1995). The original member of the genus was isolated from rancid butter by Derby and Hammer (1931), and named "*Achromobacter putrefaciens*". This taxon has undergone repeated reclassification but has settled as the genus *Shewanella* since MacDonell and Colwell (1985) created the genus based on 5S RNA sequencing. At present seven species of *Shewanella* are recognised (Table 1.1), classified according to phenotype, genotype, growth rate, whole-cell fatty acid analysis, DNA-DNA hybridisation, and phylogenetic analysis (Bowman *et al.*, 1997). *Shewanella* strains have been isolated from a wide range of habitat including marine and fresh water, anaerobic sediments, oil fields, fish, meat, and clinical specimens (Dawood and Brozel, 1998; Dhawan *et*

al., 1998; Levy and Tessier, 1998; Molin and Ternstrom, 1982; Obwuekwe *et al.*, 1981; Pickard *et al.*, 1993; Richard, 1977, Shirasaka *et al.*, 1997). The main reason why *Shewanella* can proliferate in such a range of environments is due to their ability to couple growth to the reduction of an extraordinary number of electron acceptors. To date *Shewanella* have been shown to reduce at least ten terminal electron acceptors, including nitrate, fumarate, elemental sulphur, trimethylamine N-oxide, manganese and ferric iron (Table 1.2; Moser and Nealson, 1996; Myers and Nealson, 1988;Tsapin *et al.*, 1994; Scott and Nealson, 1994).

Table 1.1. Characteristics that differentiate *Shewanella* species. **From, Bowman *et al.* (1997).**

Species	Growth at:				Production of:		
	25°C	30°C	Growth in 8% NaCl	Seawater required	Chitinase	Amylase	Ornithine decarboxylase
S. gelidimarina	-	-	-	+	+	-	-
S. frigidimarina	+	-	+	-	-	-	v
S. benthica	-	-	-	+[a]	+	-	-
S. hanedai	-	-	-	+[b]	+	+	-
S. putrefaciens	+	+	-	-	-	-	+
S. alga	+	+	+	+	-	-	+
S. colwelliana	+	+	-	+	-	+	-

Species	Fermentation of:		Dissimilatory Fe(III) reduction	Synthesis of EPA	G+C content (mol%)
	D-Glucose	N-Acetyl-glucosamine			
S. gelidimarina	-	+	+	+	48
S. frigidimarina	+	-	+	+	40-43
S. benthica	+	+	v	+	47
S. hanedai	-	-	-	+	43-44
S. putrefaciens	-	-	+	-	44-47
S. alga	-	-	+	-	53-54
S. colwelliana	-	-	ND[c]	ND	46

[a] *S. benthica* requires a vitamin(s) for growth
[b] *S. hanedai* requires yeast extract for growth
[c] ND - no data available
v – variable

As shown in table 1.2, these electron acceptors span a wide range of electron potentials from highly electropositive compounds like oxygen to more electronegative ones such as sulphite (Saffarini and Nealson, 1993). In order to understand how this respiratory plasticity is accomplished it is necessary to examine how bacterial aerobic and anaerobic respiratory chains are coupled to energy production.

Table 1.2. Electron acceptors used by *Shewanella*.TMAO, trimethylamine N-oxide; DMSO, dimethyl sulphoxide; S^0, elemental sulphur.

Electron acceptor	Midpoint potential (mV)
Oxygen	+ 820
Fe (III)	+ 770
Nitrate	+ 420
Mn(IV)	+ 380
Nitrite	+ 360
DMSO	+160
TMAO	+130
Fumarate	+ 40
S^0	- 240
Thiosulphate	- 372
Sulphite	- 516

1.2 The generation of ATP

The cell needs energy for biosynthesis, polymerisation and sometimes formation of precursor metabolites. It also needs additional energy for other purposes such as motility, bringing substrates into the cell and keeping metabolites within the cell (Neidhardt *et al.,* 1990). The total energy requirement is met through two fundamentally different biochemical reactions - substrate level phosphorylation and electron transport-linked phosphorylation.

In substrate level phosphorylation, ATP is formed from ADP by transfer of a high-energy phosphate group from an intermediate of a fueling reaction, most commonly occurring in the early stages of carbohydrate metabolism (Voet and Voet, 1990).

In a number of different modes of microbial metabolism including respiration and photosynthesis, ATP is generated by transporting electrons through a chain of carrier molecules with fixed orientation in the cell membrane (Stanier *et al.*, 1987). A theory to explain how electron transport could be coupled to the generation of ATP was first proposed by Peter Mitchell in his chemiosmotic theory. Electron transport expels protons from the cell. Because the membrane itself is impermeable to H^+ and OH^- ions, the flow of protons out of the cell creates a difference in proton concentration and electrical charge across the cytoplasmic membrane of bacteria. The sum of these is a form of potential energy called proton motive force, which can be used for a variety of purposes, including synthesising ATP from ADP.

1.3 Bacterial respiration

The ability of organisms to survive in diverse metabolic environments is a direct result of their unique ability to use several alternatives to oxygen for respiration, and in many cases to switch to and from oxygen at will according to the demands of the environment. As such, the physiological environment often determines the nature of the proteins found in bacterial electron transfer chains.

All biological electron transfer reactions occur between redox centres located in proteins. Those involved in the oxidation of organic compounds most commonly contain four different classes of molecules. Two classes, the flavoproteins and quinones, are hydrogen carriers whereas the other two, the iron-sulphur proteins and cytochromes are electron carriers.

The mitochondrial respiratory chain is probably the most extensively studied aerobic respiratory chain. It contains four membrane-bound complexes (I-IV), ubiquinone/ubiquinol and a peripheral protein, cytochrome *c* (Figure 1.1).

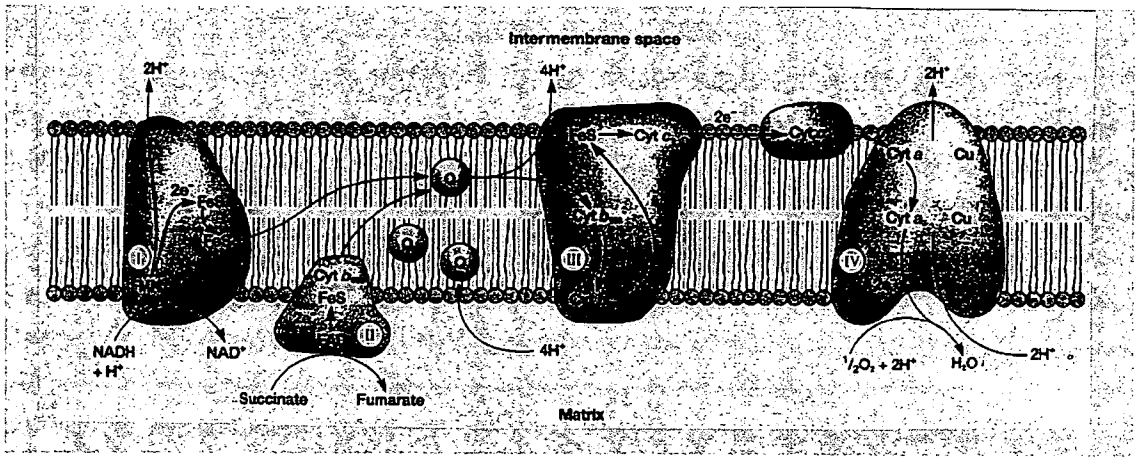


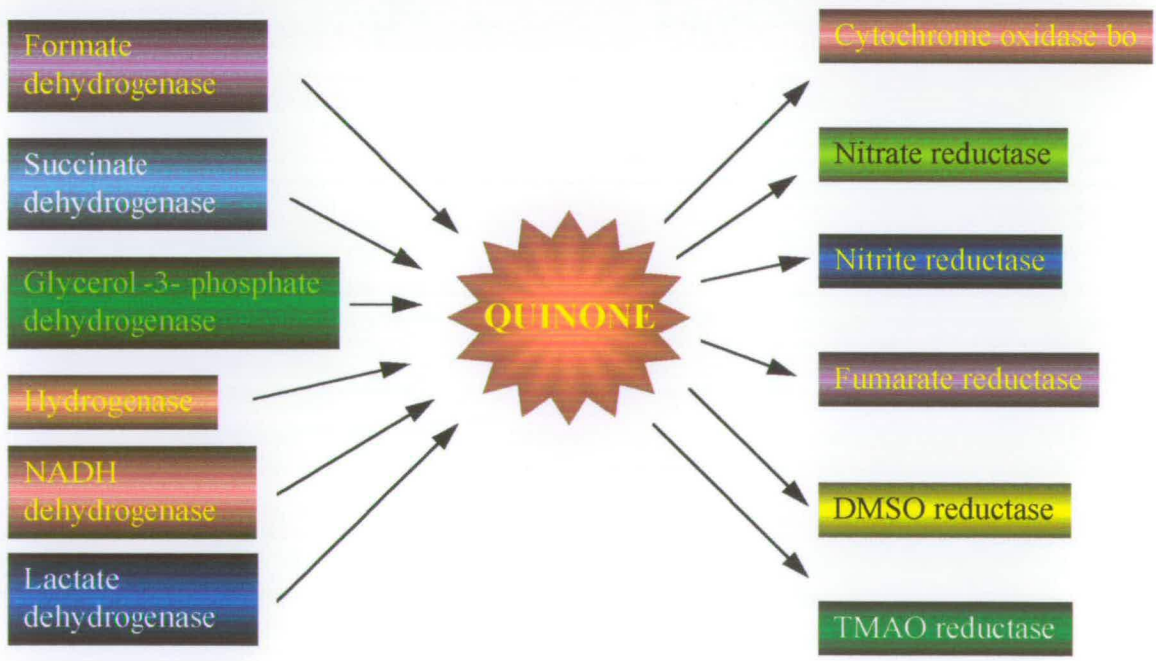
Figure 1.1 The respiratory chain of eukaryotic mitochondria. In this scheme, the carriers are organised asymmetrically within the inner membrane so that protons are transported across as electrons move along the chain. **Cyt** - cytochrome, **FeS** - non-haem iron protein, **Q** - coenzyme Q, **FAD** - Flavin Adenine Dinucleotide, **FMN** - Flavin Mononucleotide. **Prescott *et al.*, 1996**.

Studies of bacterial respiratory chains have revealed similar chains of carriers. Whilst the respiratory chains of aerobically grown bacteria and of mitochondria both contain these four types of redox carriers, it is noticeable that there are great differences in the variety of respiratory patterns displayed by bacteria. The variations in components between and within bacterial species, and between the bacteria and mitochondria, are usually attributed to either the replacement of one type of carrier with another, or the addition or deletion of specific carriers. Variations in the pattern of redox carriers can also be induced within single species by altering the growth conditions and occur principally amongst the quinones and the terminal oxidases (Morris, 1987).

1.3.1 Respiratory chains in *Escherichia coli*

Escherichia coli is one of the preferred bacteria for studies of respiratory pathways. The nucleotide sequences of all respiratory enzymes are known, and most of the enzymes have been isolated and characterised (Unden and Bongaerts, 1997). The respiratory chains of *E. coli* consist of 15 primary dehydrogenases and of 10 terminal reductases linked by quinones in the same modular arrangement observed in the mitochondrial respiratory chain. However, due to the large number of primary dehydrogenases, quinones and terminal reductases a large variability in the composition of the respiratory chains is observed. The respiratory chains are branched at the quinone level (Figure 1.2). Other potential branch points, such as diffusable cytochrome *c*, are absent.

Figure 1.2. Diversity of respiratory pathways in *Escherichia coli*. A selection of respiratory dehydrogenases and terminal oxidases. **Unden *et al.*, 1994; Unden and Bongaerts, 1997.**



Since the dehydrogenases and terminal reductases use quinones as a common substrate or redox mediator each of the dehydrogenases should be able to react with any of the terminal reductases. However, because terminal reductases as well as dehydrogenases are expressed only under specific conditions, the number of enzymes

able to interact is restricted. In addition, thermodynamic constraints preclude the cooperation of some dehydrogenases and terminal reductases.

The electron donors or acceptors of the respiratory chain enzymes have largely differing midpoint potentials, ranging from formate (- 0.43 V) to succinate (+ 0.03 V) for the donors and from oxygen (+ 0.82 V) to fumarate (+ 0.03 V) for the acceptors. *E. coli* and related enteric bacteria synthesise three different quinones, ubiquinone (UQ), menaquinone (MK) and demethylmenaquinone (DMK). During aerobic growth the major quinone is ubiquinone. Ubiquinol (the reduced form of ubiquinone) is too electropositive to operate as an electron donor for fumarate, DMSO and TMAO and the respective reductases accept electrons only from the more electronegative napthoquinones MKH_2 (reduced menaquinone) or $DMKH_2$ (reduced demethylmenaquinone) (Wissenbach *et al.*, 1990).

1.3.2. Respiratory chains of *Paracoccus denitrificans*

Escherichia coli is capable of using oxygen, nitrate, nitrite, dimethylsulphoxide, trimethylamine N-oxide and fumarate as terminal electron acceptors. However, the respiratory chains described above are relatively simple with respect to other facultative anaerobes. This may be demonstrated by analysis of the respiratory chains of *Paracoccus denitrificans. P. denitrificans,* as well as possessing *E. coli*-like respiratory chains, also has other respiratory-chain components which allow it to grow aerobically on methylamine as well as anaerobically using nitrite or nitrate as terminal electron acceptors (Ingledew and Poole, 1984). These alternative modes of growth necessitate additional redox components, therefore the respiratory chain of *P. denitrificans* is considerably more branched than that in mitochondria or *E. coli* (Figure 1.3).

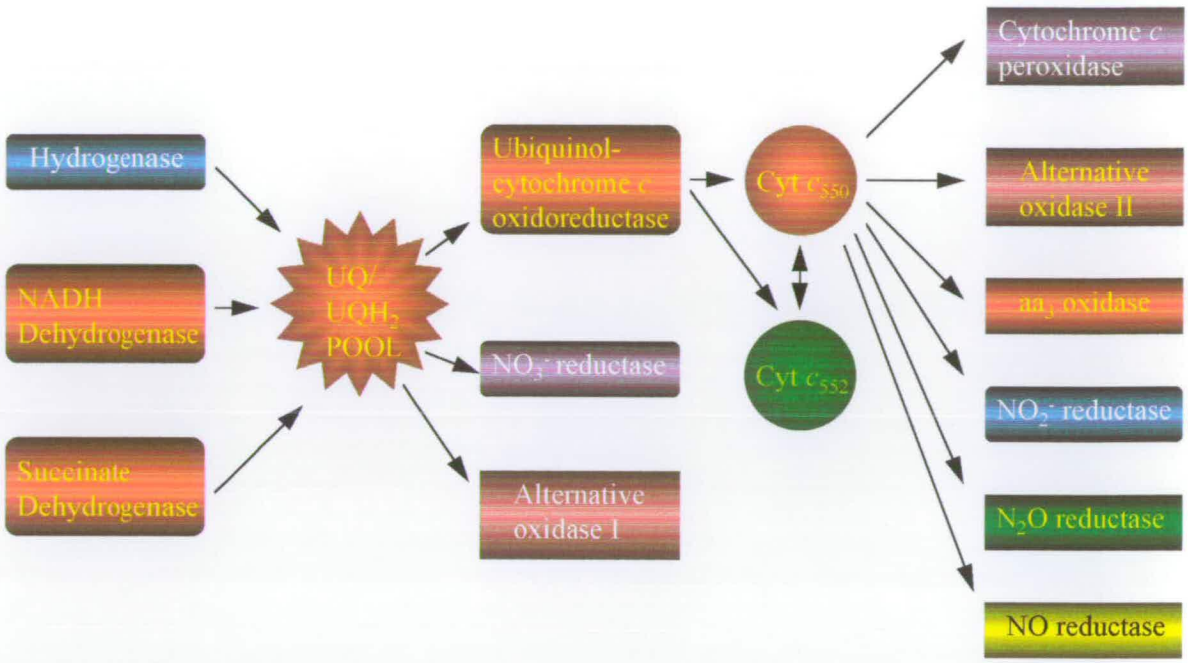


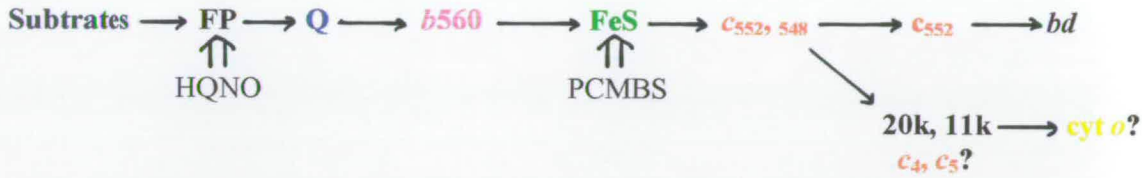
Figure 1.3. Respiratory chain of *Paracoccus denitrificans*. Arrows represent the direction of electron transport. Red coloured shapes indicate the components homologous to those from mitochondria. UQ/UQH_2 - ubiquinone/ubiquinol. **Sone and Toh, 1994.**

1.3.3 The respiratory chains of *Shewanella*

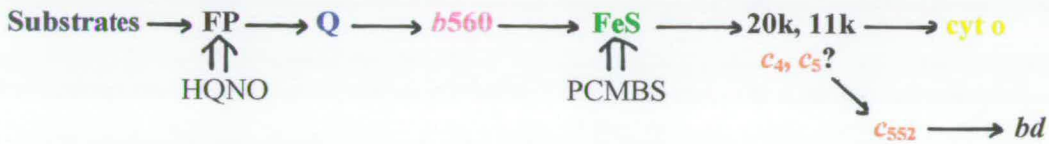
The variety of diverse environments in which *Shewanella* are found suggest that the respiratory chains might be very complex. As a result of this they have attracted much attention. Morris (1987) made preliminary investigations into the components of the respiratory chains, both aerobic and anaerobic, of *S. frigidimarina* NCIMB400, the results of which are given in figure1.4. These results, however, by no means represent the complete picture; much is still to be elucidated. For example, preliminary evidence of the involvement of respiratory *c*-type cytochromes in reductive dehalogenation of tetrachloromethane, by another *Shewanella* strain, *S. putrefaciens,* has been found (Picardal *et al.*, 1993). *S. putrefaciens* has also been shown to have the ability to grow anaerobically with Fe (III) as a sole terminal electron acceptor (DiChristina and DeLong , 1994).

Figure 1.4. Hypothetical respiratory chains of *Shewanella frigidimarina*. A and B are alternative pathways. **HQNO** - 2-*n*-heptyl-hydroxyquinoline-N-oxide; **PCMBS** - *p*-chloromercuribenzene sulphonate; Peak 8 - cytochrome c_3-like octahaem cytochrome. **Morris, 1987.**

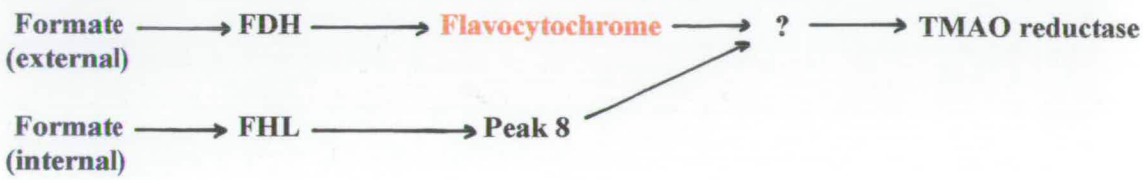
A. Hypothetical scheme for aerobic electron transport in *Shewanella frigidimarina* NCIMB400



B. Hypothetical scheme for aerobic electron transport in *Shewanella frigidimarina* NCIMB400



C. Hypothetical scheme for anaerobic respiration to TMAO in *Shewanella frigidimarina* NCIMB400



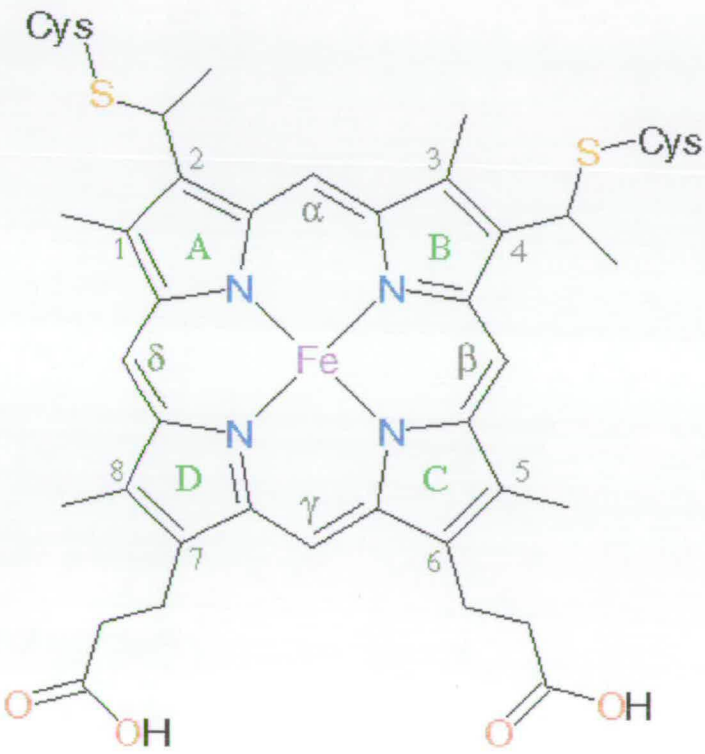
The work of Morris (1987) illustrates the central importance of cytochromes in the respiratory chains of *S. frigidimarina* ; in particular, the role of cytochromes *c*, which allow flexibility in the use of electron donors and acceptors (Pettigrew and Moore, 1987). It is important to note that these pathways are hypothetical. However, they do give an indication of the difficulty in elucidating respiratory chains in organisms such as *Shewanella*.

1.4 Cytochromes

Cytochromes form a diverse group of proteins with only a few features in common. They are coloured proteins related by the presence of a bound haem group whose iron atom changes from the ferric [Fe(III)] to the ferrous [Fe(II)] state whenever it accepts an electron (Alberts *et al.*, 1989). The haem group consists of four pyrrole

rings joined to each other by methylene bridges and the four planar N-atoms liganded to the central Fe (Figure 1.5).

Figure 1.5. The iron-porphyrin group of cytochrome *c*



Cytochromes are present in all biological oxidations which involve transport of reducing equivalents through organised chains of intermediates regardless of the ultimate oxidant (Horio and Kamen, 1970). Thus, as well as being present in systems involved in reduction of oxygen, cytochrome systems exist in facultative anaerobes, obligate anaerobes and facultative photoheterotrophs. However, they are not found in systems which exhibit primarily a fermentative anaerobic mode of metabolism involving substrate esterification.

Classically, cytochromes have been divided into four types, *a, b, c,* and *o*, based on their spectroscopic properties. This classification system is quite straightforward: the associated prosthetic haem groups are sufficiently different to give rise to distinctive optical absorption spectra, although those of the *b*- and *c*-types do overlap (Pettigrew and Moore, 1987). There is also no sequence similarity between the three classes, and therefore no ambiguity in assigning a particular sequence to a particular class (Pettigrew and Moore, 1987). More recently cytochromes have been divided into four main structural subclasses: mitochondrial *c*-type cytochromes, b_5-type cytochromes, four-α-helical cytochromes, and multihaem cytochromes (Mathews, 1993).

1.5 Cytochromes *c*

The first authentic bacterial cytochrome to be isolated and characterised was a *c*-type cytochrome from the phototrophic bacterium *Rhodospirillum rubrum* in 1953 (Meyer and Kamen, 1982). Since then the knowledge of *c*-type cytochromes has increased dramatically aided by the number of readily soluble forms amenable to experiment at the pure protein level and, more recently, the availability of genetic information.

Cytochromes *c* are defined as those cytochromes with a covalently linked haem prosthetic group. Both *a*- and *b*-type cytochromes have non-covalently attached haem. Further subdivision of the *c*-type cytochromes is important in order to understand the relatedness of the electron transport chains and organisms of which they are a part. Ambler (1980) has divided the group into three classes based on amino-acid sequences and three-dimensional structures.

1. 5. 1 Class I

The most studied type of cytochrome *c* is the group of class I cytochromes *c* implicated in electron transfer with nearly ubiquitous occurrence among eukaryotic and prokaryotic organisms. This group is characterised by possession of a single haem group located near the N-terminus and a methionine located near the C-terminus which serves as the sixth haem ligand (Morelle *et al.*, 1995; Moore *et al.*, 1982). The presence of the methionine sixth haem ligand and a histidine as the fifth haem ligand gives rise to the "695 nm band" in the ferricytochrome spectrum. These proteins are low spin and have the 550 nm band. They also tend to have high redox potentials. As a result of the widespread occurrence of the class I *c*-type cytochromes, great variability in redox potential as well as sequential and structural divergence is found due to adaptation to the different metabolisms in which they function.

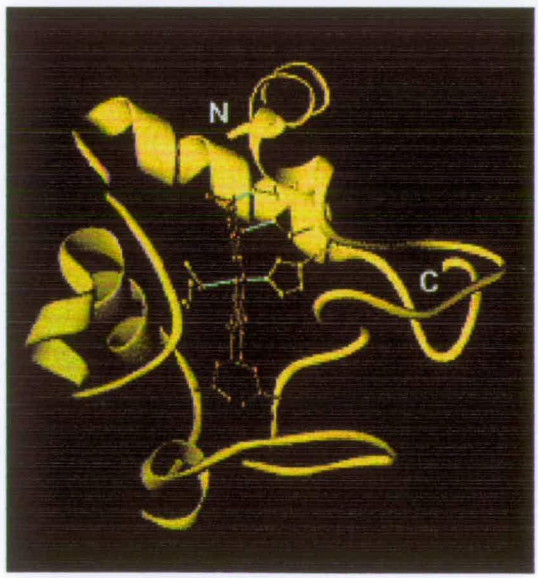
Based on the size of the molecule, this family has been broadly divided into two major groups: the large cytochromes *c* (100-130 amino acids), and the small bacterial and algal cytochromes *c* (80 amino acids). The size difference is mainly due to the

presence or absence of a large loop situated below the haems (Pettigrew and Moore, 1987).

1.5.1.1 Class Ia - Large cytochromes

The structural and sequence similarity between eukaryotic cytochromes *c* and prokaryotic cytochromes c_2, essentially from photosynthetic bacteria, has grouped these into a single subclass. This subclass has been particularly well characterised structurally and functionally. There have been high resolution X-ray studies of cytochromes *c* from tuna, horse and rice and the cytochromes c_2 from *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Rhodopseudomonas viridis* and *Paracoccus denitrificans* (Blackledge *et al.*, 1996; Sanishvili *et al.*, 1995). The crystal structure of ferrocytochrome c_2 from *Rhodopseudomonas viridis* has been refined at 1.6Å resolution (Figure 1.6). This cytochrome functions as a water soluble electron carrier in the light driven cyclic electron-transfer process in non-sulphur purple bacteria (Sogabe and Miki, 1995). *Rps. viridis* cytochrome c_2, like c_2 from *P. denitrificans*, has a very low redox potential (285 mV) compared to other bacterial cytochromes c_2 which have redox potentials around 350 mV (Sogabe and Miki, 1995).

Figure 1.6. Ribbon diagram of cytochrome c_2 from *Rhodopseudomonas viridis*. **Sogabe and Miki, 1995**



Like other cytochromes c_2 and mitochondrial cytochromes *c* the overall protein folding is well conserved. The haem environment also appears to be very similar to other cytochromes *c*. The amino acid sequences of a variety of cytochromes *c* are known. Despite sequence identity of less than 40%, the overall folding is well conserved throughout the class.

1.5.1.2 Class Ib - Small cytochromes

The smaller class I cytochromes consist of a more diverse set of proteins typified by *Pseudomonas* cytochrome c_{551}, *Chlorobium* cytochrome c_{555}, cytochromes c_4 and c_5 and also cytochrome c_6 from cyanobacteria. The amino acid sequence varies considerably more than within the Ia group reflecting the high diversity of different bacterial and algal metabolisms.

However, the structural determinations carried out so far suggest that the Ib class retains the core structural elements and polypeptide found in class Ia (Bersch *et al.*, 1995; Blackledge *et al.*, 1995).

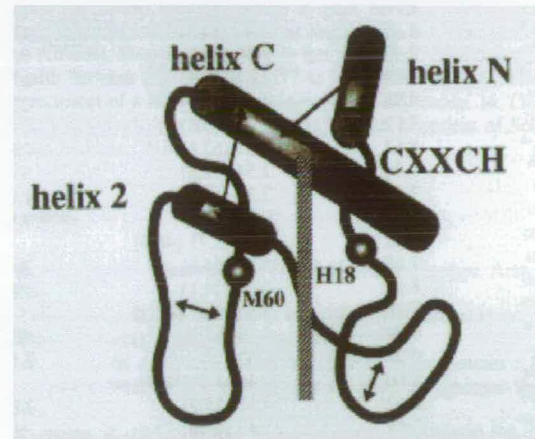
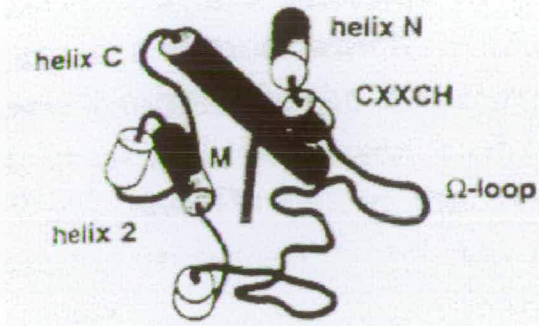


Figure 1.7. Cytochrome c_{555} from *Chlorobium limicola.* **Morelle *et al.*, 1995.**

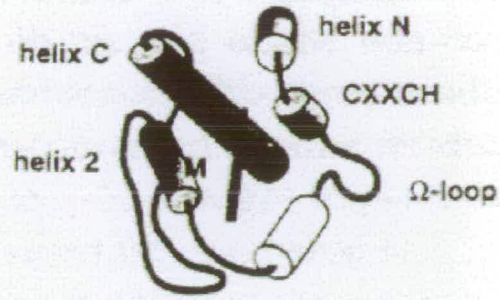
The best characterised member to date is the cytochrome c_{555} from the anaerobic green sulphur bacterium *Chlorobium limicola*, which appears to have a central role in mediating electron transfer between the various soluble and membrane bound cytochromes in this organism. It functions as a soluble carrier of electrons between the cytochrome bc_1 complex and the photosynthetic reaction centre in addition to acting as a terminal electron acceptor in the oxidation of reduced sulphur compounds (Morelle *et al.*, 1995); *Chlorobium* c_{555} accepts electrons from flavocytochrome *c*-sulfide dehydrogenase, and the two proteins form an electrostatically stabilised complex. Based on NMR assignment, the features of *Chl. limicola* cytochrome c_{555} have been determined (Figure1.7; Morelle *et al.*, 1995). Several NOEs (Nuclear Overhauser enhancements) suggest a contact between the N- and C-terminal helices, a widely conserved feature in cytochromes *c*. In figure 1.8, the topological models of several other class I cytochromes *c* are shown for comparison. As in all cytochromes *c*, the three conserved α-helices are well defined in *Chl. limicola* cytochrome c_{555}. The common cytochrome *c* fold can be confirmed using only the NMR data (Bersch *et al.*, 1995).

Figure 1.8. Selected models of small class I *c*-type cytochromes.
A, B - **Bersch *et al.*, 1995;** C,D – from the **Swissprot** online database at ExPASy.

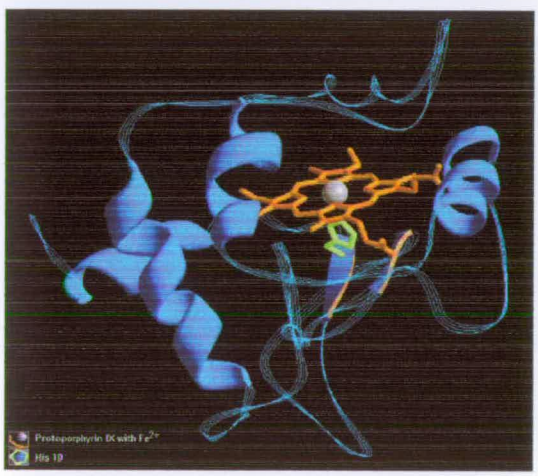
A. Yeast cytochrome *c*



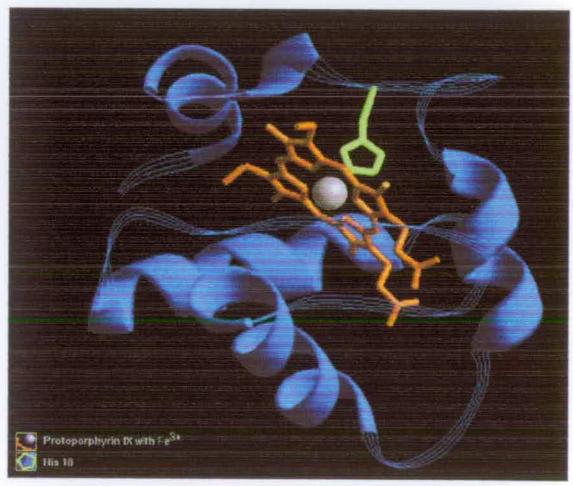
B. *Erwinia halophila* cytochrome c_{551}



C. Cytochrome c_{550} precursor from *Paracoccus denitrificans*.



D. *Pseudomonas aeruginosa* cytochrome c_{551}



1.5.1.3 Function of class I cytochromes *c*

The availability of high-resolution 3D-structures of cytochromes *c* has caused immense interest with respect to the relationship between structure and function. However, the functional roles of class I cytochromes have not been well documented with the exception of c_2 (Ia) and c_6 (Ib) (Sone and Toh, 1994). Cytochromes c_2 and c_6 provide electrons to haem\Cu-type oxidases as well as mediating electron transfer between the bc_1(*bf*) complex and the photosynthetic reaction centre or photosystem I (Sogabe and Miki, 1995; Frazão *et al.*, 1995). In *R. sphaeroides*, cytochrome c_2, under denitrifying conditions, carries out electron transfer between cytochrome bc_1

and the terminal three steps of this pathway: nitrite reductase, NO reductase, and N_2O reductase (Gans *et al.*, 1996). When cultured in the dark, expression of cytochrome c_2 in *R. sphaeroides* has also been shown to be derepressed by DMSO, even though it is unlikely to be a component of the respiratory chain under these conditions (Donohue *et al.*, 1986).

P. denitrificans, grown under anaerobic, denitrifying conditions, or methylotropically in the presence of oxygen contains substantially more cytochrome c_{550} than cells grown aerobically on multi-carbon sources (Stoll *et al.*, 1996). However, despite these findings, and also analysis of mutant strains with the structural gene interrupted (Van Spanning *et al.*, 1995), no obligatory role for cytochrome c_{550} has been determined. It has been suggested that cytochrome c_{550} of *P. denitrificans* acts as an electron carrier, which can, if necessary, be substituted by another protein. This highlights the difficulty in assigning a function to cytochromes within this class.

Initial comparisons of structures of class I cytochromes have revealed the presence of a common core of conserved helices, around which the various surface loops appear to be species dependent. The haem environment necessary for the electron transfer and the conformational changes implicated in the electron transfer process have been investigated in detail by comparison of high-resolution structures of oxidised and reduced cytochrome *c*. A network of conserved residues in the vicinity of the haem-cleft has been implicated in the function of the molecule.

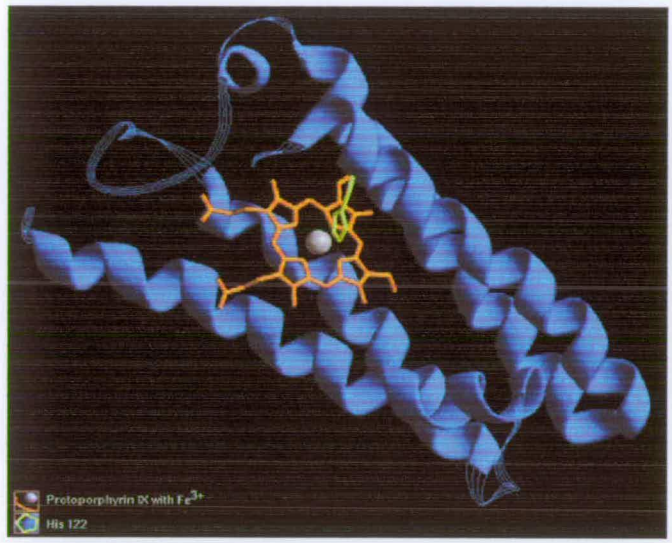
One anomaly to the class I type of *c*-type cytochromes has recently been described by Klarskov *et al.* (1998). Cytochrome c_{551} from *Chl. limicola*, strain Tassajara, is a soluble dimeric protein containing identical subunits of about 30 kDa, with a single haem binding site. It functions as the electron acceptor for a thiosulphate oxidising enzyme. The total length of the polypeptide chain is more than twice that of the class I cytochromes, and the haem binding region is nearer the C-terminal end of the protein. The occurrence of a disulfide bridge is another feature of this cytochrome, shared only with the small cytochromes c_5 and the oxygen binding haem protein (SHP) from *Rhodobacter sphaeroides*. The far-UV circular dichroism spectrum

indicates 40 % α-helix and 25 % β-sheet secondary structure. This contrasts with the predominantly α- helical structure of the class I *c*-type cytochromes described above. Klarskov *et al.* (1998) suggest that this cytochrome c_{551} is representative of a new class of *c*-type cytochrome.

1.5.2 Class II

Comparatively little is known about class II cytochromes *c* compared to class I proteins, although eleven class II cytochromes *c* have been sequenced (Moore *et al.*, 1982). They are characterised by the covalent attachment of their haem near the C-terminus. Class II cytochromes have been divided into two subclasses which are very different in terms of absorption spectra and haem coordination. Only two of the class II proteins sequenced are known to be low spin, cytochromes c_{556}, the remainder have a more complex spin-state designation and are called cytochromes *c*'. The cytochromes *c*' have been well characterised structurally. They generally consist of two identical 14 kDa subunits (Caffrey *et al.*, 1995). Each subunit possesses a four-helix bundle structural motif (Figure 1.9). As a consequence of ligand binding properties the cytochromes *c*' serve as simple dimeric models of cooperative interactions in proteins; the cytochromes *c*' exhibit cooperative binding of carbon monoxide not too dissimilar to the cooperative binding exhibited by myoglobin and haemoglobin.

Figure 1.9. Three-dimensional structure of a single subunit of cytochrome *c*' from *Rhodospirillum molaschianum*. ***From*, Swissprot online database at ExPASy.**



The biochemical functions of class II cytochromes *c* have not yet been established although they have been suggested to operate in electron transport chains, either as simple electron transfer proteins or as terminal oxidases.

1.5.3 Class III

Class III *c*-type cytochromes are characterised by their size, tetrahaem nature (Ambler, 1991), and low-redox potential due to the bis-histidinyl ligation of the haem iron atoms. They have no structural similarity to the other cytochrome *c* classes (Bruschi *et al.*, 1992). Three different class III cytochromes have been identified and purified from *Desulfovibrio* species - the tetrahaem cytochrome c_3, the octahaem cytochrome cc_3 and a high molecular weight (Hmc) cytochrome containing 16 haems (Cjzeck *et al.*, 1994). On the basis of amino-acid sequence alignment it has recently been proposed that they form a cytochrome c_3 superfamily (Cjzeck *et al.*, 1996). More recently, a trihaem cytochrome c_3 has been isolated from *Desulfuromonas acetoxidans* (Turner *et al.*, 1997). Sequence similarity suggests this cytochrome is structurally similar to the tetrahaem cytochromes c_3 (Coutinho *et. al.*, 1993). No X-ray structures are available for trihaem cytochromes.

The most interesting of the class III cytochromes is cytochrome c_3. It is found in all sulphate reducers belonging to *Desulphovibrio* species and other genera such as *Desulphomicrobium* and *Desulphobulbus* (Morais *et al.*, 1995). A small cytochrome c_3 has also been identified in *Shewanella putrefaciens* (Tsapin *et al.*, 1996).

Cytochrome c_3 is composed of a single polypeptide chain and 4 nonequivalent haem molecules, with a total molecular weight of approximately 13,000 Da (Kwoh *et al.*, 1993).

Cytochrome c_3 is of considerable structural interest, not only because of the unusually low redox potentials of the haem iron atoms in the molecule, but also because of the complex network of haem-haem co-operativity (Picarra-Pereira *et al.*, 1993). The remarkable stability of the 4 redox centres has also permitted several detailed spectroscopic studies, including the application of multidimensional nuclear magnetic resonance (NMR) spectroscopy (Coutinho *et al.*, 1993; Picarra-Pereira *et al.*, 1993; Salgueiro *et al.*, 1992), electron paramagnetic resonance (EPR) spectroscopy (Morais *et al.*, 1995; Bruschi *et al.*, 1992), and electrochemistry (Bruschi *et al.* 1992).

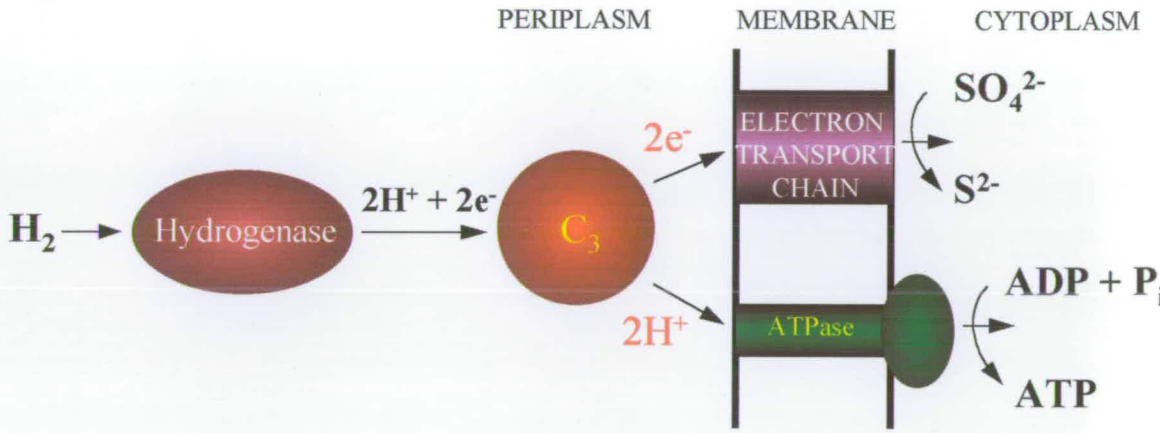
The sequences of 7 cytochrome c_3 molecules have been determined (Morais *et al.*, 1995). Alignment of these sequences suggests as few as 20% of the 107-116 residues are strictly conserved . However, analysis of the 4 known tertiary structures indicates that the overall shape is conserved and the four haem groups have a standard conformation (Figure 1.10). The X-ray data available provide an accurate picture of the arrangement of haems as well as of the amino acids surrounding the haems and interacting with redox partners (Schlereth *et al.*, 1993). Each haem exhibits an individual redox potential in the range -200 to -400 mV, and has the same His-His iron atom axial ligands but with a different local environment, explaining the different redox potentials (Bruschi *et al.*, 1992).

Figure 1.10. Structure of *Desulfovibrio* cytochrome c_3. **Mathews, 1993.** Haem groups are given in green; α-helix highlighted in yellow.

1.5.3.1 Function of cytochrome c_3

The periplasmic location of cytochrome c_3 together with the observed activation in its presence of electron transfer reactions catalysed by hydrogenase, points towards a common role of cytochrome c_3 as a specific coupling to this enzyme (Cjzeck *et al.*, 1994; Schlereth *et al.*, 1993). A comprehensive study of cytochrome c_3 from *Desulfovibrio vulgaris* has been carried out by Xavier *et al.* (Matias *et al.*, 1993; Louro *et al.*, 1996, 1997, and 1998). As a result of kinetic modeling studies a model for the role of cytochromes c_3 from these sulphate reducing bacteria has been proposed (Figure 1.11).

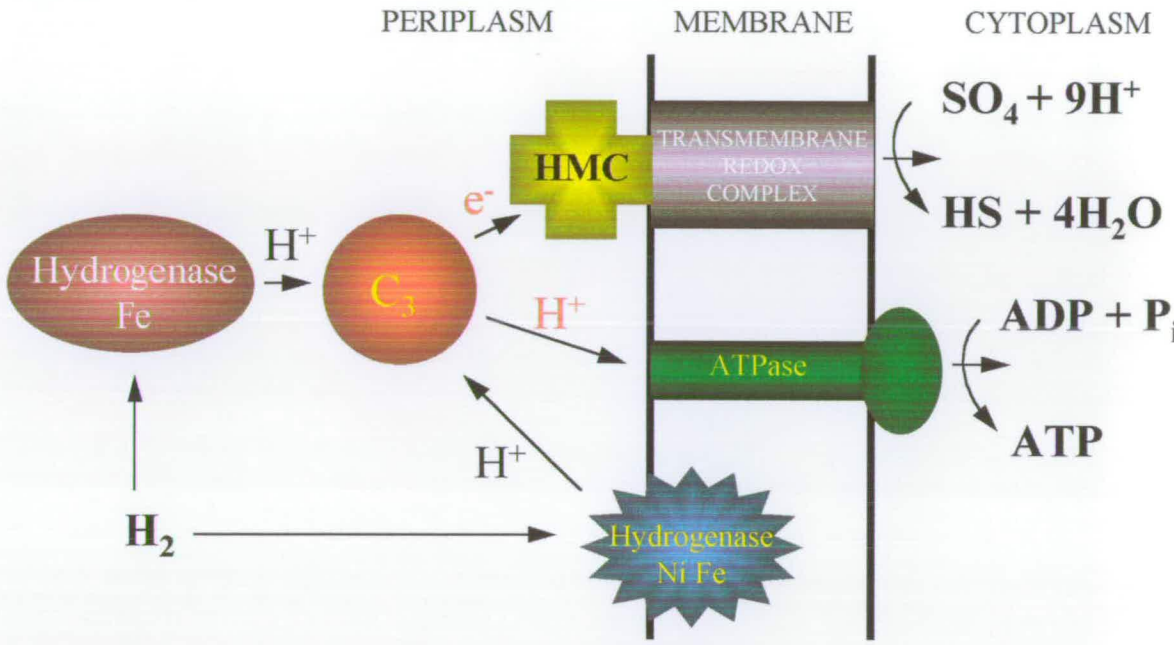
Figure 1.11 . "Proton thruster" model for Cytochrome c_3 in *Desulphovibrio vulgaris*. **Louro *et al.*, 1997.**



In this model, cytochrome c_3 acts as a charge separation device; the protein receives low energy protons and high energy electrons from periplasmic hydrogenase, and transfers them to the electron transport system and an ATPase, as low energy electrons and high energy protons respectively. The cytochrome is crucial in aiding the hydrogenase to maintain optimum activity at physiological pH.

When hydrogen is used as the electron donor for *Dv. vulgaris*, it is oxidised by either a periplasmic [Fe]hydrogenase or the membrane-bound [NiFe]hydrogenase (Figure 1.12), which then donate these electrons and protons to cytochrome c_3. The deenergised electrons are then passed from cytochrome c_3 to the high molecular weight cytochrome c_3 (Hm*c*), which in turn, transfers them through the transmembrane redox complex to the cytoplasmic reduction of sulphate. The energised protons are used for ATP synthesis

Figure 1.12. Model for the role of cytochrome c_3 and Hmc from *Desulphovibrio vulgaris* during growth on H_2. **Catarino, *pers. com.***.



Site directed mutagenesis has been used to probe the conserved residues within several cytochromes c_3 (Saraiva *et al.*, 1996; Aubert *et al.*, 1997). However no effort has been made to determine the physiological role(s) of cytochromes c_3 through genetic means.

1.5.4. Flavocytochromes *c*

Under anaerobic conditions, the most common *c*-type cytochrome found in *Shewanella frigidimarina NCIMB400* is flavocytochrome c_3. As their name suggests, flavocytochromes *c* contain two types of prosthetic group: flavins and haems. The haem groups are similar to those of the class III *c*-type cytochromes. Flavocytochrome c_3 from *S. frigidimarina* consists of a single polypeptide chain of 571 amino acids (63,800 Da) which appears to form two domains: an N-terminal cytochrome domain containing 4 *c*-type haem binding motifs (C-x-x-C-H), and a flavin domain, which binds flavin adenine dinucleotide (FAD) non-covalently (Pealing *et al.*, 1992). Sequence comparisons with other class III *c*-type cytochromes show no sequence similarity apart from the characteristic haem binding motifs. Other

flavocytochromes *c* have been isolated from *Chromatium vinosum*, *Chlorobium thiosulfatophilum* and from *Pseudomonas* species, but these differ in their physiochemical properties from *S. frigidimarina* flavocytochrome c_3. *C. thiosulfatophilum* flavocytochrome *c* functions a as a soluble sulphide dehydrogenase. The flavocytochrome c_3 from *S. frigidimarina* is a soluble, unidirectional fumarate reductase (Morris *et al.*, 1994: Gordon *et al.*, 1998).

1.6 Cytochromes *c* from *Shewanella frigidimarina*

Investigation of the respiratory chain of *S. frigidimarina* NCIMB400 has shown the periplasm to contain several *c*-type cytochromes (Table 1.3), the most abundant in anaerobic conditions being flavocytochrome c_3 (Number 6, table 1.3; Easter *et al.*, 1983; Morris *et al.*, 1990).

Table 1.3. The *c*-type cytochromes of *Shewanella frigidimarina*. Crude experimental data from **Morris *et al.*, 1990**.

	Molecular Weight (kDa)	Redox Potential (mV)	α-peak (nm)	Growth Conditions
1	8.5	+ 217	551	Aerobic, Anaerobic
2	11	ascorbate reducible	552	Aerobic, Anaerobic
3	84	- 204, -320	552.3	Aerobic, Anaerobic
4	100	low	550	Anaerobic
5	110	low	552	Anaerobic
6	40	- 200, -286	551	Anaerobic
7	32	- 180, -300	552	Anaerobic
8	20	ascorbate reducible	552	Aerobic, Anaerobic
9	110	low	552	Anaerobic

In order to extend our understanding of the nature of the respiratory chains in *S. frigidimarina*, two of the small *c*-type cytochromes, cytochrome c_3 and cytochrome c_5 (Table 1.3, numbers 7 and 2, respectively), have been the focus of a more comprehensive study.

1.6.1 Cytochrome c_3 from *Shewanella frigidimarina* NCIMB400

A small, acidic, low potential tetrahaem cytochrome has been isolated from the periplasm of *Shewanella frigidimarina* NCIMB400 (Gordon *et al.*, unpublished). The protein was seen to contain four haem prosthetic groups with low potential (- 250 to 0 mV) (Pike, 1998). These properties are shared with the cytochromes c_3 from

sulphate-reducing bacteria (section 1.5.3), and as a result this protein was designated as a cytochrome c_3.

The gene encoding this cytochrome has been cloned and sequenced. *Shewanella* cytochrome c_3 consists of 86 amino acid residues with a predicted molecular weight of 11, 780 Da, including the four attached haem groups.

Comparison of the amino acid sequence with sequences in the available databases has revealed several interesting results; the only clear similarity is seen with the cytochrome domain of flavocytochrome c_3, also purified from *S. frigidimarina* (Pealing *et al.*, 1992), with a cytochrome c_3 from the phototrophic bacterium *H1R*, and with a similar cytochrome c_3 recently isolated from *S. putrefaciens* (Tsapin *et al.*, 1996). These sequences are aligned in figure 1. 13.

Figure 1.13. Amino acid sequence alignment of cytochrome c_3 from *S. frigidimarina* (S.f c_3) with cytochrome c_3 from the phototrophic bacterium *H1R* (*H1R* c_3), a partial sequence of flavocytochrome c_3 from *S. frigidimarina* (Fcc$_3$), corresponding to the tetrahaem cytochrome domain, and the N- terminal sequence of cytochrome c_3 from *S. putrefaciens* MR-1 (MR1 c_3). Haem attachment sites are shown in red.

```
S.f c3 ADETLAEFHV EMGGCENCH. ADGEPSKDG. AYEFEQCQSC HGSLAEMDDN
H1R c3 AD.VLADMHA EMSGCETCH. ADGAPSEDG. AHEAAACADC HGGLADMEAP
Fcc c3 AD.NLAEFHV QNQECDSCHT PDGELSNDSL TYENTQCVSC HGTLAEVAET
MR1 c3 ADQKLSDFHA ESGGCESCH
```

The relationship with cytochromes c_3 from *Desulphovibrio* species is much more distant and no significant similarity is observed outwith the haem attachment sites. The close relationship between cytochrome c_3 and the cytochrome domain of flavocytochrome c_3 indicates a relatively recent duplication of this sequence. The physical and spectroscopic properties between the two are also very similar, however, the relationship between their functions is unclear. Flavocytochrome c_3 is a soluble fumarate reductase (Pealing *et al*, 1992; Gordon *et al.*, 1998), but the function of cytochrome c_3 is unknown.

Shewanella sp. are the first organisms capable of aerobic growth found to contain cytochrome c_3 and its presence raises interesting questions about the architecture of the respiratory chains of this organism. In addition, *Shewanella sp.* are the only known facultative anaerobes also capable of dissimilatory growth with elemental sulphur as an electron acceptor.

Many respiratory proteins in bacteria are encoded in operons and coexpressed with functionally related proteins. Sequencing of the region surrounding the structural gene of cytochrome c_3 from *S. frigidimarina* has however not revealed any cotranslated proteins likely to be functionally related (Gordon *et al.*, unpublished).

1.6.2 Cytochrome c_5 from *Shewanella frigidimarina* NCIMB400

It was during purification of flavocytochrome c_3 that a reduced cytochrome was noticed which eluted before a salt gradient was added to the ion-exchange column. This cytochrome had a molecular weight of 20 kDa. This small, class Ib, cytochrome has been purified and its N-terminal sequence shown to be similar to those of cytochromes c_5 from other bacteria (F.Allen, unpublished work) (Figure 1.14).

Figure 1.14. N-terminal amino acid sequence alignment of cytochromes c_5. Putative haem binding sites are shown in red. Lower case letters represent uncertainties in the determination. Ps - *Pseudomonas*; men. - *mendocina*; stutz - *stutzeri;* Az.vin. - *Azotobacter vinelandii*; Org. H-1-R. - Organism H-1-R.

```
Ps.aeruginosa          ...RSGEDIVGKTCNTCHGTGLLGAPKVGDKAEW...
Ps. men.ch110          ...RSADDIIAKHCNACHGAGVLGAPKIGDTAAW...
Ps.stutz.Stanier-221   .rqgdavvakFCTAchgsgIINAPKvgdsaawk..
Az.vin.Strain0         ...RSGDDVVAKYCNACHGTGLLNAPKVGDSAAW...
Org. H-1-R             ...QEGEAVYNKACQTCHAMGIAGAPKLNDAAAW...
S. frigidimarina         XEGEAIYNKACQVCHSMGVAGDPKVcDLaD...
```

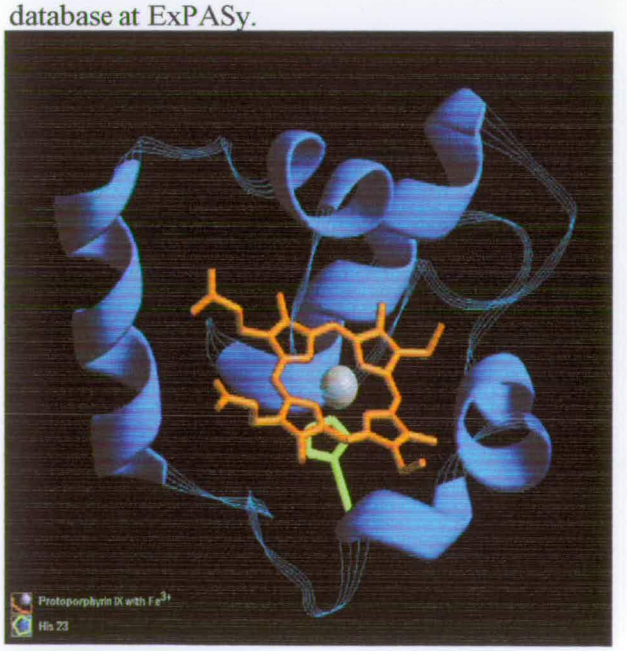
A feature of cytochromes c_5 sequenced so far is a characteristic second C-X-X-C site which has no haem bound. This pair of cysteines forms a disulphide link in

Azotobacter cytochrome c_5. Their presence is highly unusual, the only other known *c*-type cytochromes with more cysteine pairs than are necessary are bovine mitochondrial cytochrome c_1 and yeast mitochondrial cytochrome *c*. The reason for the conservation of these cysteine pairs is not known.

A further recurring structural feature is a ragged N-terminus; this gave rise to the fear that cytochrome c_5 may be a fragment of a larger protein derived by endogenous protease action (Ambler, 1974; Lenhoff and Kaplan, 1956). This worry has now been resolved following isolation of the cognate gene (this work).

The majority of available information on cytochrome c_5 is based on the protein from two organisms from which it has been isolated: *Azotobacter vinelandii* and *Pseudomonas mendocina*. In these bacteria, cytochrome c_5 occurs as a dimer of a monohaem cytochrome with a molecular weight of approximately 10 kDa and the α-peak of the ferrocytochrome is shifted to the red near 555 nm (Tissières, 1956; Tissières and Burris, 1956; Swank and Burris, 1969; Ambler and Taylor, 1973; Meyer and Kamen, 1982). Investigation of purified cytochrome c_5 from *S. frigidimarina* has shown it to be a high potential cytochrome with a mid-point potential of + 274 mV (F.Allen, unpublished results).

Figure 1.15. 3D structure of cytochrome c_5 from *Azotobacter vinelandii*. *From*, the **Swissprot** online database at ExPASy.



Preliminary crystallographic data for *Azotobacter* cytochrome c_5 first appeared in 1968 (Stout, 1968). Since then the crystal structure has been determined at 2.5Å resolution (Carter *et al.*, 1985). Figure 1.15 shows the 3-dimensional model of *Azotobacter vinelandii* cytochrome c_5. The structure of cytochrome c_5 has been the subject of a critical discussion as the protein used for the structure determination behaves as a monomer in solution but appears to form a dimer in the crystal (Moore and

Pettigrew, 1990). So it is not clear whether the crystal structure really corresponds to the biochemically relevant form (Bersch *et al.*, 1995). On comparison with the models shown in figure 1.8 (Page 14), it can be noted that *Azotobacter* cytochrome c_5 and *Erwinia halophila* cytochrome c_{551} possess an almost identical folding of the protein with regards to the haem. Sequence alignments, together with the structural data, high redox potential, isoelectric points, Soret bands and electron-spin resonance spectroscopy, firmly place cytochrome c_5 as a class Ib cytochrome *c*.

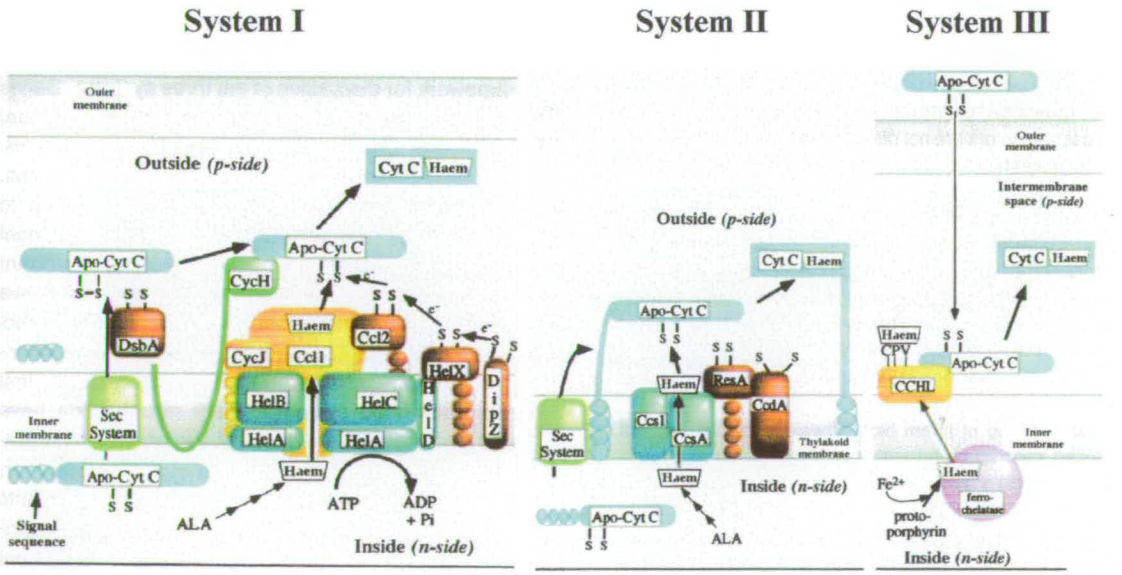
Despite the knowledge of amino acid sequence and structure, the biochemical function of cytochrome c_5 is not known. Historically cytochrome c_5 has been linked with cytochrome c_4, due to their copurification (Swank and Burris, 1969). In *Azotobacter* the respiratory chain is branched and inhibition experiments indicate that electrons flow from cytochrome c_4 to cytochrome c_5 and then to cytochrome o (Swank and Burris, 1969). However, redox titrations with cytochromes c_4 and o indicate that cytochrome o does not function as a terminal oxidase in *Azotobacter* (Carter *et al.*, 1985).

The expression of cytochromes c_4 and c_5, in *Azotobacter vinelandii*, has been shown to be up-regulated under N_2 starvation (Rey and Maier, 1997). Subsequent gene disruption experiments have indicated that the role of these cytochromes is to provide respiration at intermediate (5 to 10%) and low (below 5%) O_2 tensions.

1.7 Cytochrome *c* biogenesis

As described, all *c*-type cytochromes contain a sequence motif consisting of the amino acids C-X-X-C-H, in which the cysteine residues serve to bind a haem group covalently. Also, all bacterial *c*-type cytochromes reside in the relatively oxidising environment of the periplasm, either in solution, membrane bound, or tethered by non-covalent protein-protein interactions. The mechanism(s) by which these proteins reach their destination and attach haem covalently has been the subject of much speculation (Ramseier *et al.*, 1991; Goldman *et al.*, 1996; Grove *et al.*, 1996; Crooke and Cole, 1995; Beckman *et al.*, 1992; Thöny-Meyer *et al.*, 1994; Metheringham *et al.*, 1995). Molecular biological studies of the biogenesis of *c*-type cytochromes have so far elucidated three different systems (Figure 1.16; Kranz *et al.*, 1998). System I requires specific proteins encoded by at least nine genes and has been found in most Gram negative bacteria. System II is characteristic of Gram positive bacteria, cyanobacteria, and chloroplasts and involves at least four genes. Finally, system III, found mainly in mitochondria from fungi, requires only a single protein called cytochrome *c*-haem lyase.

Figure 1.16. Working models of the three distinct systems for cytochrome *c* biogenesis. **Kranz *et al.*, 1998.**



Several common principles and requirements are shared between the three systems, the first of which is the transport of apocytochrome *c* to its site of function before

haem attachment. As haem is not synthesised at the site of function, haem must be transported or diffuse to this compartment. Secondly, at the assembly site haem must find the two apocytochrome *c* cysteinyl residues and, in a stereospecific manner, the thiol groups must attack carbons on the vinyl side of the haem. Thus the cysteines must be reduced.

For the purpose of this review only system I, that proposed for Gram negative bacteria, will be described in further detail. Here two functional subpathways are introduced: a putative haem delivery pathway, and a periplasmic thioreduction-oxidation pathway.

1.7.1 Haem delivery

The proteins required for haem delivery are encoded by *helABCD*, *ccl1*, and *cycJ*. Compelling evidence has been provided to suggest that haem travels through HelABCD to Ccl1 where it is tethered to the periplasmic surface with the haem-vinyl side chains exposed (Figure 1.6).

1.7.2 Periplasmic thioreduction-oxidation

The second subpathway that makes up system I cytochrome *c* biogenesis is apocytochrome presentation and thioreduction. The proteins required for this pathway are encoded by *ccl2*, *helX* and *cycA*.
Firstly apocytochrome *c* travels through the general secretory pathway to reach the periplasm (Figure 1.16; Sambongi *et al.*, 1996; Thöny-Meyer and Künzler, 1997). Following the secretion of apocytochrome *c*, the periplasmically oriented and membrane-bound Ccl1/Ccl2/CycH/HelX proteins are proposed to continue the assembly process of the oxidised apocytochrome. Ccl2 is proposed to reduce the cysteinyl residues of apocytochrome *c* while presenting it to the haem, which is tethered to Ccl1. Subsequently the thioredoxin-like protein HelX reduces oxidised Ccl2. It is also thought that DipZ, a general thioreduction protein, may reduce HelX either directly or indirectly (Crooke and Cole, 1995). A further requirement for cytochrome *c* biogenesis is that the apocytochrome cysteinyl residues are initially

oxidised after secretion through general secretory components; a role for the periplasmic oxidoreductase DsbA may be to directly oxidise cytochrome *c* cysteinyl residues as they are secreted (Metheringham *et al.*, 1995).

One of the topics for future investigation of cytochrome c biogenesis is the specificity that is built into the systems. Eaves *et al.* (1998) have recently identified a multihaem cytochrome, NrfA, which contains four typical C-X-X-C-H motifs with haems attached through the system I pathway, but also a fifth motif, C-W-S-C-K. A second set of genes that includes *nrf*EFG (similar to the *ccl*1, *ccl*2, and *cyc*H genes) was subsequently identified to be required for ligation of haem to this motif. It seems plausible that a subset of dedicated proteins have evolved specifically to recognise this unique C-W-S-C-K motif.

Further goals include a deeper understanding of haem delivery. It seems likely that even more factors involved in cytochrome c biogenesis will be discovered over the forthcoming years.

1.8 Regulation of respiratory pathways

One of the interesting questions which arises from examining complex respiratory pathways, such as those observed in *S. frigidimarina*, is how such an organism regulates the synthesis and activity of these electron transport networks. Are all networks constitutively expressed? Are some pathways more efficient than others?

1.8.1 Electron acceptor hierarchy

Facultative anaerobes such as *Escherichia coli* can grow under either aerobic or anaerobic conditions, obtaining energy from respiration or fermentation. Choice of process depends on the availability of oxygen or alternative electron acceptors, the relative rate of electron flux, or the fermentability of the carbon source. These pathways are not equivalent with respect to the ATP yield and *E. coli* has developed successive layers of regulatory mechanisms to exploit the environmental energy sources to its greatest profit. In oxidative phosphorylation, the key to optimising energy generation lies in channeling electron transport from donors to acceptor(s) so that the voltage drop is maximal (Iuchi and Lin, 1993). In *E. coli*, if oxygen is present it will be used in preference to all other acceptors. However, in the absence of oxygen, the acceptor with the next most positive mid-point redox potential will be used, in this case nitrate. For example, nitrate ($E^0 = + 420$ mV) is favoured over fumarate ($E^0 = + 30$ mV). As a result, four hierarchical levels of regulation exists in *E. coli*. The highest priority level, (I) is represented by aerobic metabolism, followed by nitrate respiration (II) and fumarate, TMAO, and DMSO-respiration (III), and the lowest level fermentation (IV) (Unden and Bongaerts, 1997; Unden *et al.*, 1994).

Similar electron acceptor hierarchy has been observed in *Bacillus macerans*, but for other bacteria different regulatory criteria might be valid. In *Thiosphaera* denitrification is not repressed by oxygen. Also, in the gut bacterium *Wolinella succinogenes*, nitrate does not repress fumarate and co-respiration of both acceptors is observed. Indeed, the most electro-negative acceptor polysulphide with the lowest

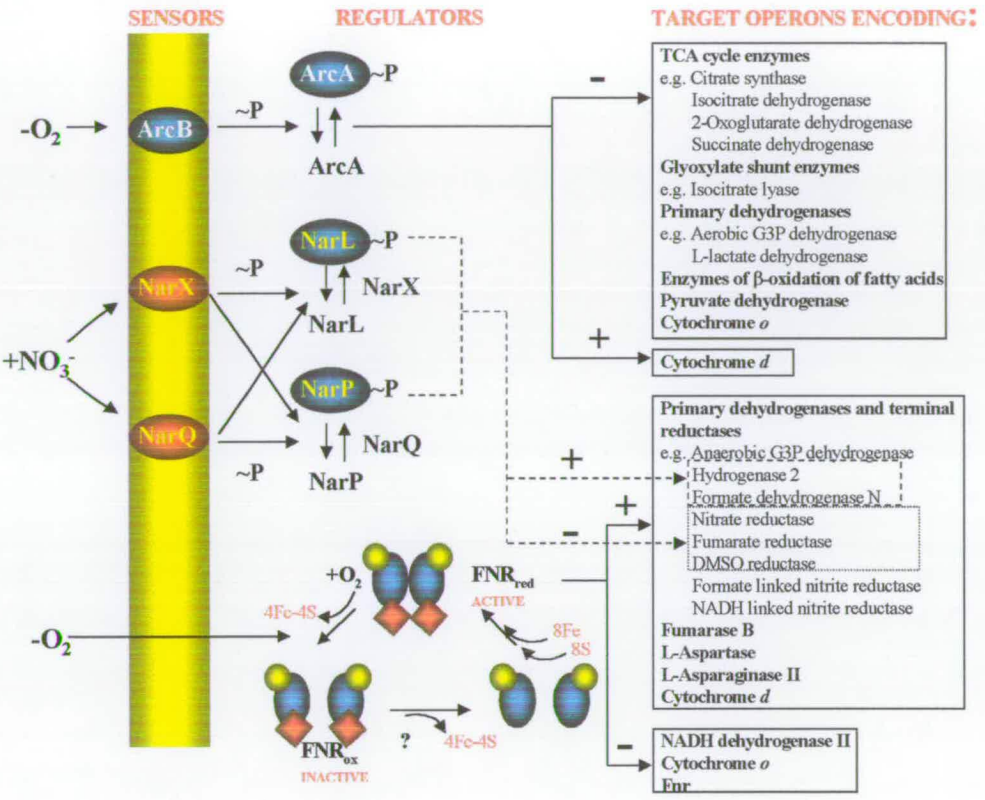
ATP yield appears to repress fumarate and nitrate respiration (Macy *et al.*, 1986; Unden *et al*, 1994).

To effectively express and control the various metabolic pathways, gene regulation must be capable of allowing the cell to interpret various environmental signals, which ultimately lead to an appropriate metabolic response. A general picture of how different respiratory pathways are expressed has been built up over the last 30 years: switching between pathways involves the actions of both specific and global transcriptional regulatory proteins. Four broadly acting systems have so far been described from *E. coli* (Figure 1.17).

1.8.2 ArcA-ArcB

In the presence of oxygen, many genes of aerobic function become highly expressed. In the absence of oxygen, mutants in *arc*A or *arc*B also express these genes. It was characterisation of these mutants in *a*erobic *r*espiration *c*ontrol that led to the recognition of the ArcA-ArcB system (Iuchi and Lin, 1988; Iuchi *et al.*, 1989; Iuchi *et al.*, 1990; Iuchi and Lin, 1991). The ArcA and ArcB proteins are members of a large family of bacterial two-component sensor-regulator proteins. Two component systems consist of a sensor protein, typically a transmembrane protein responsive to environmental changes, and a regulatory protein which, in most cases, is a cytosolic transcription factor (Gross *et al.*, 1989; Stock *et al.*, 1989). In the ArcA-ArcB system, ArcA is a cytosolic transcriptional regulator which consists of an N-terminal receiver domain and a C-terminal DNA binding domain. ArcB is a transmembrane protein with a periplasmic sensor domain and a C-terminal cytoplasmic transmitter domain.

Figure 1.17. Pleiotropic regulators in *Escherichia coli*. **Unden and Bongaerts, 1997; Iuchi and Lin, 1991.**



On the basis of *in vitro* phosphorylation experiments, a model for the ArcA-ArcB signal transduction process has been proposed (Iuchi, 1993). On stimulation His292, a conserved residue in the receiver domain of ArcB, undergoes autophosphorylation. This phosphate group is then transferred intramolecularly to a conserved Asp residue, Asp576, also in the receiver domain of ArcB. Upon rephosphorylation of His292, ArcA may be activated. A conserved Asp in the receiver domain of ArcA is phosphorylated by ArcB-PP (the double phosphorylated ArcB protein), which results in activation (Iuchi and Lin, 1992; Iuchi, 1993). ArcA, thus activated, either represses or activates its target operons. The target operons have been identified as operons encoding enzymes of the tricarboxylic acid cycle, the glyoxylate shunt, the pathways for β-oxidation of fatty acids, and the aerobic electron transport chain (Figure 1.17). In general, ArcA represses its targets under anaerobic conditions. The cytochrome *d* (*cyd*) operon is an exception; it is activated by ArcA. ArcA not only regulates operons involved in aerobic metabolism, but it is also necessary for the phenotypic expression of the F plasmid. The sensor for the presence of the F plasmid

is not ArcB but another transmembrane protein, CpxA (Ronson *et al.*, 1987). ArcA has also been demonstrated to be required for anaerobic regulation of the *p*yruvate *f*ormate *l*yase operon (*pfl*; Sawers, 1993).

The stimulus for ArcB is still not known. Iuchi *et al.* (1991) demonstrated that mutants blocked in both cytochromes *d* and *o* are greatly impaired in sensing anaerobiosis. This suggests that the direct stimulus is not oxygen itself, but an electron carrier in reduced form that accumulates because of oxygen deficiency. More recently, accumulation of certain metabolites, such as D-lactate and acetate, has been shown to enhance the phosphorylation of both the transmitter and receiver domains of ArcB *in vitro* (Iuchi *et al*, 1994).

1.8.3 Nar X/L and NarQ/P

The preferential induction of nitrate reductase over other terminal reductases whose function is less energetically rewarding is attributable to another set of two-component regulatory systems, NarX/L and NarQ/P. Nar X/Q and NarL/P are homologous sensor-regulator pairs responsive to nitrite and nitrate. The NarX/L system is responsible for ensuring the preferential use of nitrate over all other terminal electron acceptors during anaerobic respiration, whereas the NarQ/P system controls nitrite induction of several operons (Sun *et al.*, 1996).

On the basis of amino acid sequence similarity, NarL was recognised as the regulator element of a two-component regulatory system in which NarX serves as the sensor element. NarX, upon stimulation by nitrate and molybdate, undergoes autophosphorylation. Thus activated NarX~P activates NarL by phosphorylation. Activated NarL acts as both a positive and a negative regulator of gene expression. NarL represses operons encoding terminal reductases for terminal electron acceptors of lower redox potential than nitrate, such as *frd*ABCD (for fumarate reductase) (Figure 1.17). NarL stimulates the nitrate reductase and formate dehydrogenase operons. Of the two response-regulator proteins, NarL and NarP, only NarL binds DNA to control nitrate induction of nitrate reductase.

NarL binds to the consensus site TACYYMT, called the NarL-heptamer. NarL heptamers exhibit great diversity with respect to number, location, orientation, and spacing, frequently being arranged in tandem (Unden and Bongaerts, 1997; Darwin *et al.*, 1997). Groups of these heptamers may be found at +1, -100 or -200 with respect to the transcription initiation site. In contrast to NarL, NarP only binds to heptamer sequences organised as an inverted repeat with a 2bp spacing (7-2-7 sites; Darwin *et al.*, 1997).

1.8.4 FNR

For *E. coli*, the metabolic responsiveness to changes in oxygen tension is mediated in part by the anaerobic regulatory *fnr* (for *f*umarate and *n*itrate *r*eduction) gene product that controls the transcription of a number of genes, acting both positively and negatively in response to reduced oxygen tension (Tyson *et al.*, 1993, 1994; Darwin *et al.*, 1997; Sawers *et al.*, 1988). FNR is a further example of a pleiotropic regulator, but differs from the above systems in that it consists of both sensor and regulator domains in a single polypeptide. FNR is a typical prokaryotic gene regulator with a helix-turn-helix DNA binding motif. It shows significant homology to the CRP (*c*yclic AMP *r*eceptor *p*rotein) of *E. coli* which effects catabolite repression. FNR contains an N-terminal extension with four cysteine residues, three of which are part of a cluster of four conserved and essential cysteine residues in the protein.

The mechanism by which FNR senses oxygen, or redox potential, has been a matter of debate for a number of years. The most recent model suggests that the conserved cysteine residues serve as ligands for an oxygen-responsive [4Fe-4S] cluster (Figure 1.17; Kennedy, 1998; Unden and Bongaerts, 1997). Under anaerobic conditions FNR exists as a dimer with each monomer containing a single [4Fe-4S] cluster. In this state the protein is active and binds to DNA. On exposure to oxygen, however, the [4Fe-4S] clusters are converted to [2Fe-2S] clusters, the dimer "breaks" into its constituent monomers, and the protein is inactive. The stimulus which causes the removal of the [2Fe-2S] clusters from FNR (Figure 1.17) is still not known. Indeed the use of oxygen as the stimulus for FNR activation has still not been proven. Unden *et al.* (1990) demonstrated that it is not oxygen *per se* that activates FNR, but rather

that the function of FNR is controlled by a redox potential of E_0= + 400 to 440 mV. Redox changes have also been suggested to be responsible for regulation of photosynthetic gene expression (Horne *et al.*, 1996; Zeilstra-Ryalls and Kaplan, 1996).

Enzymological studies with *fnr* mutants and strains containing multiple copies of the *fnr* gene have indicated that under anaerobic conditions, FNR activates a variety of terminal reductases such as the fumarate, nitrate and nitrite reductases, and also represses cytochrome *o* oxidase, one of the NADH dehydrogenases and its own synthesis (Figure 1.17).

Footprinting studies with purified FNR have confirmed that it is a site-specific DNA binding protein (Sharrocks *et al.*, 1991). From the FNR binding sites of several FNR regulated promoters a consensus of dyad symmetry (TTGAT-N_4-ATCAA) has been derived. The sequence is composed of two half sites with a strictly conserved spacing of four nucleotides. Each halfsite is assumed to bind one FNR monomer. In most cases the FNR-box is centered around - 41.5 with respect to the transcriptional start site of positively regulated promoters. In examples where FNR inhibits transcription, FNR binds to a site which either overlaps or is adjacent to the sigma 70 recognition sequence and presumably blocks the binding of RNA polymerase (Iuchi and Weiner, 1996).

Homologues of *E. coli* FNR have been identified in a variety of taxonomically diverse bacterial species including *Alcaligenes eutrophus*, *A. denitrificans*, *A. faecalis*, *Azotobacter vinelandii*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, a number of *Pseudomonas* species, and the Gram positive *Bacillus macerans* (Ramos *et al.*, 1995; Schirawski and Unden, 1995; Spiro, 1994; Sun *et al.*, 1996;Van Spanning *et al.*,1995; Wu *et al.*, 1997; Zeilstra-Ryalls and Kaplan, 1995)

1.8.5 Other regulators

In addition to the global regulatory systems described above, a number of other more specific regulators may be found in *E. coli*. A comprehensive account of all identified regulators is outwith the scope of this review, however one of the most relevant is AppY (acid polyphosphatase) involved in the anaerobic induction of hydrogenase I, quinoloxidase AppBC, acid phosphatase, and some other genes. In the regulation of many genes by oxygen or nitrate, a regulation due to growth rate or growth phase is also imposed (Unden and Bongaerts, 1997). The target genes responding to the regulators are found in diverse and unrelated metabolic pathways.

1.8.6 Interaction of regulatory systems

Although the various regulatory systems operate independently, each shares some of its target operons with the others. As a result, most of the target genes are either directly or indirectly controlled by more than one global system so that they are expressed in a finely tuned, sophisticated manner. This allows expression to be adapted to best suit a specific set of conditions such as the combined availability of oxygen, carbon sources, and iron. Dual control of cytochrome *d* and cytochrome *o* genes by the ArcA and FNR regulatory proteins provides an effective way to microaerobically control the synthesis of the two alternative aerobic respiratory chains (Tseng *et al.*, 1996). Targets for other regulator proteins overlapping FNR sites are suspected to aid fine-tuning of expression of a number of anaerobically induced genes including the *pfl* operon (Kaiser and Sawers, 1997; Cotter *et al.*, 1997).

1.8.7 Anaerobic gene regulation in *Shewanella*

Regulation of anaerobic respiration has not been studied in detail in *Shewanella* species. However, anaerobic growth on fumarate, nitrite, Fe(III), TMAO, DMSO, thiosulphate, and sulphite by *S. putrefaciens* MR1, has been shown to be dependent on a gene designated *etr*A, which encodes a protein very similar to FNR (Safarini and Nealson, 1993). EtrA shows 73.6% identity to *E. coli* FNR, and has also been shown to complement an *E. coli fnr* null mutation.

Anaerobic growth on nitrate and Mn(IV) is independent of *etr*A and hybridisation experiments have shown that *S. putrefaciens* may contain a second *etr*A gene (Saffarini and Nealson, 1993).

More recently, Tn5 mutagenesis was used to produce pleiotropic respiratory mutants of *S. putrefaciens*, which were incapable of growth on any electron acceptor other than oxygen. Respiratory functions were restored using specific cloned fragments, one of which is chromosomally located while the other is plasmid borne; neither hybridizes to either *etr*A or *fnr* (Saffarini *et al.*, 1994). The sequence of the genes encoded on the complementing fragments has not yet been determined.

1.9 Aims

The aims of the work in this thesis are:

1. To clone and sequence the structural gene of cytochrome c_5 from *Shewanella frigidimarina* NCIMB400.
2. To investigate the physiological function of cytochrome c_5.
3. To investigate the physiological function of cytochrome c_3.
4. To develop an overexpression system for cytochrome c_5.

Chapter 2

Materials and Methods

2.1 Bacterial strains, phage and plasmids

2.1.1 Bacterial Strains

Strain	Genotype	Reference
Azotobacter chroococcum Strain 6B	Wild type *Azotobacter chroococcum strain 6B*	*Lab Stock*
Azotobacter vinelandii Strain O	Wild type *Azotobacter vinelandii* strain O	*Lab Stock*
Shewanella frigidimarina		
ACAM591	Wild type *Shewanella frigidimarina,* type strain ACAM 591	*Bowman* et al. *(1997)*
NCIMB400	Wild type *Shewanella frigidimarina,* NCIMB400	*N.C.I.M.B.*
NCIMB400 rif^r	Spontaneous rifampicin resistant strain	*Lab stock*
EG301	NCIMB400 Rif^r, Δfcc$_3$::ahp Km^r	*E.H.J.Gordon, Ph.D Thesis*
AH301	EG301 Δ*c*3::ΩSm Sm^r	*This Work*
AH401	NCIMB400 Rif^r, Δc_3::ΩSm Sm^r	*This work*
AH500	NCIMB400 harbouring pAH534	*This work*
AH501	NCIMB400 Rif^r Δc_5::ΩSm Sm^r	*This work*
AH601	EG301 Δc_5::ΩSm Sm^r	*This work*
AH701	AH401 Δc_5::ΩSm Sm^r	*This work*
AH801	AH301 Δc_5::ΩSm Sm^r	*This work*
Shewanella putrefaciens		
MR1	Wild type *Shewanella putrefaciens* MR1	*Lab stock*

Escherichia coli

B834	F⁻*ompT* *hsd*$S_B(r_B^- m_B^-)$ *gal dcm met* (DE3)	*Denjumukhametov* et al. *(1998)*
BL21 (DE3)	E. coli *B F⁻ dcm ompT hsdS(r_B- m_B-) gal λ(DE3)*	*Stratagene™*
BL21(DE3)pLysS	E. coli *B F⁻ dcm ompT hsdS(r_B- m_B-) gal λ(DE3) [pLysS Cam^r]*	*Miroux and Walker* (1996)
BL23	F^+, prototropic *str*R, λ-	*Lab stock*
BW313	*thi pro hsd*R *hds*M^+ *rec*A, chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7) *dut ung thi*-1 *rel*A *spo*T1/F' *lys*A	*Kunkel and Roberts (1987)*
DH5α	*sup*E44 Δ*lac*U169(ϕ80 *lac*Z ΔM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	*Lab stock*
JM109 (DE3)	*e14⁻(McrA⁻) rec*A1 *sup*E44 *end*A1 *hsd*R17(*rk⁻ mk*$^+$) *gyr*A96 *rel*A1 Δ(*lac-pro*AB) F'[*tra*D36 *pro*$^+$AB *lac*I^q *lac*ZΔM15] *thi-1 mcr*B^+ *λ(DE3)*	*Stratagene™*
K12	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 λ⁻e14⁻ Δ(*lac-pro*AB) [F' *tra*D36 *pro*A^+B^+ *lac*I^q Δ(*lac*)M15]	*New England Biolabs*
NF1	*K12ΔH1, Δtrp, LacZ⁻*, λ*Nam*7, *Nam*53, cI857, ΔH1.	*Stanley and Luzio (1984)*
POP2136	*F⁻*, *end*A, *thi*, *hsd*k, *mal*T, *mal*PQ, λcI857	*Xue* et al. *(1996)*
S17-1	*thi*-1 *thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu Km^r	*Simon* et al *(1994)*
SM10	*LacY1 leuB6 mcrB*$^+$ *supE44 thi-1 thr-1 tonA21 F-* RP4-*2 (Tc::Mu)*	*Lab stock*
TG1	*sup*E *hsdδ5 thi* Δ(*lac-proAB*) *F'*[*traD36 proAB*$^+$ *lac*I^q *lac*ZΔM15] *thi*-1 *mcr*B^+	*Lab stock*

***Pseudomonas aeruginosa* P6009**	Wild type *Pseudomonas aeruginosa* P6009	*Lab stock*
***Pseudomonas fluorescens biotype* B**	Wild type *Pseudomonas fluorescens biotype* B	*Lab stock*
***Pseudomonas mendocina* CH11**	Wild type *Pseudomonas mendocina* CH110	*Lab stock*
***Pseudomonas putida* GB-1**	Wild type *Pseudomonas putida* GB-1	*Lab stock*

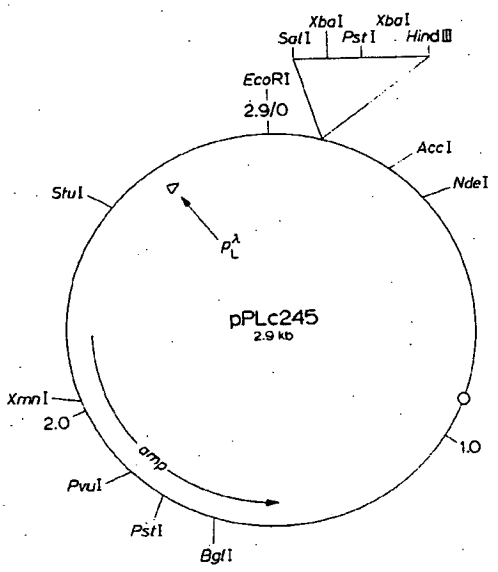
2.1.2 Plasmids

Plasmid	Description	Reference
pAH3	5634bp *S. frigidimarina* insert cloned into pTZ18r	*This work*
pAH501	1089bp PCR fragment containing *scy*A cloned into pTZ18r	*This work*
pAH502	520bp PCR fragment containing *scy*A cloned into pTZ18r	*This work*
pAH503	808bp PCR fragment containing *scy*A cloned into pTZ18r	*This work*
pAH504	1089bp PCR fragment containing *scy*A cloned into pKS-	*This work*
pAH505	1089bp PCR fragment containing *scy*A cloned into pSK-	*This work*
pAH506	4kb *Acc*I clone containing *scy*A cloned into pTZ18r	*This work*
pAH510	pAH501 containing ΩSm cassette in *Psh*A1 site	*This work*
pAH511	pAH501 containing ΩSm cassette in *Psh*A1-*Sex*A1 sites	*This work*
pAH512	2089bp insert from pAH510 cloned into pJQ200KS	*This work*
pAH513	1884bp insert from pAH511 cloned into pJQ200KS	*This work*
pAH520	pAH501 with *Eco*RI site engineered into (start)	*This work*
pAH521	pAH501 with *Nde*I site engineered into (start)	*This work*
pAH530	*scy*A cloned into pJLA503	*This work*
pAH531	*scy*A cloned into pT7-7	*This work*
pAH532	*scy*A cloned into pET11a	*This work*
pAH533	*scy*A cloned into pET19	*This work*
pAH534	*scy*A cloned into pMMB503	*This work*
pEG720	*cctA*::ΩSm fragment cloned into pJQ200KS	*E.H.J.Gordon (199*
pEG801	1700bp fragment containing *cctA* cloned into pTZ18r	*E.H.J.Gordon (199*
pTZ18/19r	General purpose phagemid cloning vector	*Rokeach* et al. *(198*
pSK$^{+/-}$	Bluescript general purpose phagemid cloning vectors	*Stratagene®*
pKS$^{+/-}$	Bluescript general purpose phagemid cloning vectors	*Stratagene®*
pJQ200KS/SK	Suicide vector for Gram negative bacteria	*Quandt and Hynes (1993)*
pHRP309	Broad host range *lac*Z transcriptional fusion vector	*Parales and Harwo*
pHRP310	Broad host range *lac*Z transcriptional fusion vector	*(1993)*
pHRP317	Broad host range *lac*Z transcriptional fusion vector	" " "
pKK223-3	Expression vector	*Brosius and Holy (19*
pRC23	Expression vector	*Crowl* et al. *(1985)*
pMMB503	Expression vector – see figure 2.1	*Overbye Michel* et a *(1995)*
pJLA503	Expression vector – see figure 2.1	*Kudo* et al. *(1983)*

pET11a	Expression vector	*Novagen™*
pET20	Expression vector	*Novagen™*
pT7-7	Expression vector	*Tabor and Richard (1985)*

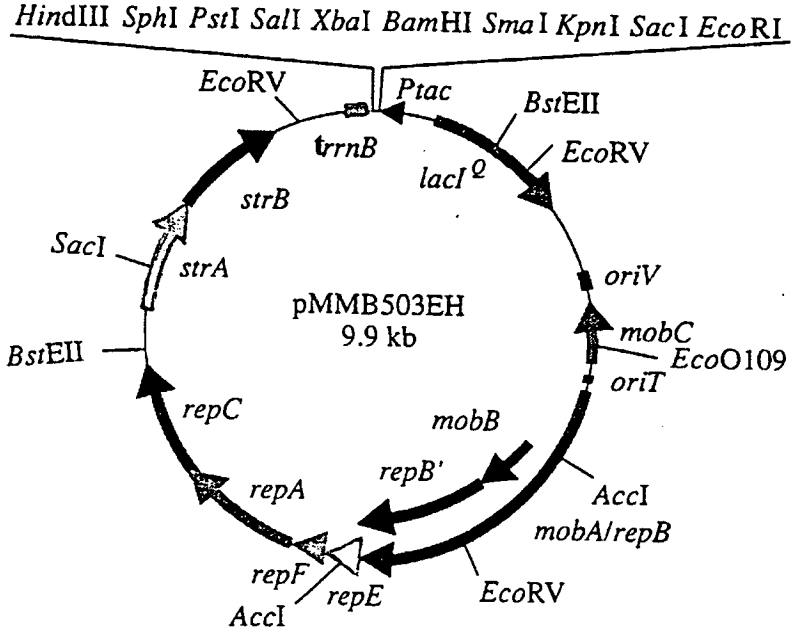
Figure 2.1. Selected plasmid maps. **A.** pPLc245, Expression vector upon which plasmid pJLA503 is based. **Kudo *et al.*, 1983**. **B.** pMMB503, broad host range expression vector for use in *Shewanella*. **Overbye Michel *et al.*,1995.**

A.



pJLA503 contains a unique cloning site for *Nde*I for expression of non-fused genes. Other unique promotor-distal cloning sites are *Bam*HI, *Eco*RI, *Sal*I, and *Sph*I.

B.



2.1.3 Bacteriophage

Phage	Description	Source/Reference
M13K07	Helper phage for single stranded DNA production	*Vieira and Messing (1987)*

2.1.4 Oligonucleotides

Oligo	Sequence 5'-3'	Function
N3187	CggAATTCgARggNgARgCNAT	**PCR**
N3188	CAggATCCACYTTNggRTCNCCNGC	**PCR**
P3928	CggAATTCgARggNgARgCNATHAYAA	**PCR**
MPF	CAggATCCgATgATAgCACTggCATgg	**PCR**
MPR	CggAATTCCCTgCATAgggTCgAgATAAg	**PCR**
MC5F	CAggATCCgACATAAACTCAATCgAg	**PCR**
MC5R	CggAATTCTgTCAgTCAACgTTCAg	**PCR**
c5prim	ACTTTAggRTCACCAgC	**Labelling**
OLIGO	gAAggTgAAgCTATTTATAAYAAAgCTTgTCAAgTTTgTCAT	**Probe**
P3929	AARgCNTgYCARgTNTg	**Probe**
M13	gTAAAACgACggCCAgT	**Sequencing**
c5seq	CCgCAATATTTCTCgTAC	**Sequencing**
c51	CAgCATgggTgTggCTg	**Sequencing**
c52	TgACATACTTgACACgC	**Sequencing**
c5nde1	ggAACATTgCATATgAAAAAACTg	**SDM**
c5ecorI	ggAACAgAATTCATgAAAAAACTg	**SDM**
c5stop	gCTAAgTAAgCTTACTggATCCAAAAACCgg	**SDM**

Oligonucleotides were synthesised on an Applied Biosystems DNA synthesiser model 380A. Positions of four-fold degeneracy were incorporated into the oligonucleotide by mixed-base synthesis, adding all four nucleotides at the coupling step.

2.2 Media and Growth Conditions

2.2.1.1 Luria-Bertani medium (LB)

Per litre:

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

For agar plates 1.5% agar was added.

2.2.1.2 S.O.C

Per litre:

Bacto-tryptone	5 g
Bacto-yeast extract	5 g
NaCl	10 mM
KCl	2.5 mM
$MgSO_4$	10 mM
$MgCl_2$	10 mM
Glucose	20 mM

2.2.1.3 Terrific Broth

Per litre:

Bacto-tryptone	12 g
Bacto-yeast extract	24 g
Glycerol	4 ml
KH_2PO_4	2.31 g
K_2HPO_4	12.54 g

2.2.1.4.1 *Shewanella frigidimarina* minimal medium. Modified from Myers and Nealson (1988), with reference to Lee *et al.* (1977).

Component	Final concentration
$(NH_4)_2SO_4$	9.0 mM
K_2HPO_4	5.7 mM
KH_2PO_4	3.3 mM
$NaHCO_3$	2.0 mM
$MgSO_4$	1.01 mM
NaCl	0.2 mM
EDTA	67.2 µM
$CaCl_2$	485 µM
H_3BO_3	56.6 µM
$FeSO_4$	5.4 µM
$CoSO_4$	5.0 µM
$NiCl_2$	3.87 µM
$NaMoO_4$	1.5 µM
$NaSeO_4$	1.26 µM
$MnSO_4$	1.04 µm
$ZnSO_4$	0.2 µM
L-Serine, or	20 µg/ml
Vitamin free Casamino acids	0.02%

Where appropriate the following supplements were added:

Electron Donor	Final concentration
Acetate	15 mM
Formate	15 mM
Lactate	15 mM
Succinate	15 mM
Hydrogen	15 % v/v

Electron Acceptor	Final concentration
Dimethylsulphoxide	2ml x stock (SIGMA)
Fe(III)Citrate	50 mM
Fe(III)Chloride	50 mM
Fe(III)Sulphate	50 mM
Fumarate	5 mM
Glycine	5 mM
KNO_2	10 mM
KNO_3	10 mM
MnO_2	30 mM
$NaNO_2$	3 mM
$NaNO_3$	15 mM
$NaSO_3$	10 mM
$Na2S_2O_3$	10 mM
$(NH_4)_2SO_4$	2.5 mM
Pyruvate	30 mM
Trimethylamineoxide	25 mM

2.2.1.4.2 *Shewanella frigidimarina* minimal medium. (Paul Dobbin, UEA)

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
$MgSO_4.7H_2O$	0.25 g
$CaCl_2.2H_2O$	0.1 g
Trace elements	50 ml
Amino acids	100 µl

Where **trace elements** solution:

Per 2 litres:

Na_2EDTA	1 g
H_3BO_3	0.14 g
NaCl	0.0234 g
$FeSO_4.7H_2O$	0.06 g
$CoCl_2.6H_2O$	0.05 g
$NiCl_2.6H_2O$	0.05 g
$NaMoO_4$	0.04 g
SeS_2 (SeO_2)	0.01 g
$ZnSO_4.7H_2O$	0.02 g
$CuSO_4.5H_2O$	0.002 g
$MnSO_4.4H_2O$	0.02 g

And **Amino Acids,**

Per 100 ml:

0.02 g Arginine
0.02 g Glutamate
0.02 g Serine

pH adjusted to 7.4.

Carbon source and electron acceptor were added as above (2.2.1.4.1), where appropriate.

2.2.1.4.3 Defined minimal medium (pH7.4). (Myers and Nealson , 1988)

Component	Final concentration
$(NH_4)_2SO_4$	9.0 mM
K_2HPO_4	5.7 mM
KH_2PO_4	3.3 mM
$NaHCO_3$	2.0 mM
$MgSO_4$	1.01 mM
$CaCl_2$	0.485 mM
H_3BO_3	67.2 µM
NaCl	10.0 µM
$FeSO_4$	5.4 µM
$CoSO_4$	5.0 µM
$Ni(NH_4)_2(SO_4)_2$	5.0 µM
Na_2MoO_4	3.87µM
Na_2Se0_4	1.2µM
$MnSO_4$	1.26µM
$ZnSO_4$	1.04µM
$CuSO_4$	0.2µM
L-Arginine HCl	20µg/ml
L-Glutamine	20µg/ml
DL-Serine	40µg/ml

Carbon source and electron acceptor were added as above (2.2.1.4.1), as appropriate.

2.2.2 Long term storage

For long term storage of strains a single colony was used to inoculate liquid medium. Once the OD_{600} reached 0.6, 0.15 ml of sterile glycerol was added to 0.85 ml of the bacterial culture. The suspension was vortexed to evenly disperse the glycerol, the vial snap-frozen in liquid nitrogen and stored at -80°C.

2.2.3 Antibiotics

Where appropriate antibiotics were added as follows:

Antibiotic	Stock solution	Working concentration
Ampicillin	50 mg/ml	100 µg/ml
Kanamycin	10 mg/ml	50 µg/ml
Streptomycin	10 mg/ml	10 µg/ml
Rifampicin	10 mg/ml	10 µg/ml

All antibiotics were made up in sterile double distilled water and filter sterilised using a 22 µm filter with the exception of rifampicin, which is made up in ethanol.

2.2.4 Growth conditions

Unless otherwise stated, bacterial cultures were incubated as follows:

Bacterial strain	**Growth temperature**
Azotobacter	23°C
Escherichia coli	37°C
Pseudomonas	30°C
Shewanella	23°C

Aerobic cultures were incubated in containers with no more than 1/5 volume of culture media. Anaerobic cultures were grown in sealed containers with rubber stoppers.

For anaerobic incubation of plate cultures, an Oxoid (Fisher microbiology) gas generating pack was used in anaerobic jars to generate an atmosphere of 90% H_2/ 10% CO_2. Alternatively, anaerobic jars were flushed with N_2 or N_2/H_2, then sealed.

2.3 Solutions

2.3.1 Alkaline lysis buffer

NaOH	0.2 M
SDS	1 %

2.3.2 Denaturing Solution

Per litre:

NaCl	86.66 g
NaOH	20 g

2.3.3 Denhardt's Solution (100 x)

Bovine Serum Albumin	2 %
Ficoll	2 %
Polyvinylpyrollidone	2 %

2.3.4 DNA Loading Buffer (10 x)

Bromophenol blue	0.25 %
Ficoll 400	15 %

2.3.5 DNA Sequencing gel (6 % acrylamide)

Per 60 ml:

Urea	25.2 g
Protogel (30 % acrylamide; 0.8% bis-acrylamide)	12.5 ml
10 x TBE	6 ml
ddH_2O	to 60 ml
10 % APS	140 µl
TEMED	140 µl

APS and TEMED added immediately before pouring the gel.

2.3.6 Neutralising Solution

NaCl	1.5 M
Tris-HCl, pH7.2	0.5 M
EDTA	1 mM

2.3.7 Pre-hybridisation solution

SSC	6 x
Denhardt's solution	5 x
SDS	0.5 %
Denatured salmon-sperm DNA	0.5 mg

2.3.8 SDS-PAGE

Formulations for SDS-PAGE separating and stacking gels:

	Separating gel 0.375 M Tris, pH 8.8			**Stacking gel 0.125 M Tris, pH6.8**
	18 %	**12 %**	**7.5 %**	**4.0 %**
Protogel (see 2.3.5)	60 ml	40.0 ml	25.0 ml	1.3 ml
ddH_2O	13.5 ml	33.5 ml	48.5 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	25.0 ml	25.0 ml	25.0 ml	-
0.5 M Tris-HCl, pH 6.8	-	-	-	2.5 ml
10 % (w/v) SDS	1.0 ml	1.0 ml	1.0 ml	100 ml
10% APS	500 µl	500 µl	500 µl	50 µl
TEMED	50 µl	50 µl	50 µl	10 µl
Total	100 ml	100 ml	100 ml	10 ml

APS made fresh and added with TEMED immediately before pouring gel.

2.3.9 SDS-PAGE loading buffer (2 x)

Per 100ml:

1 M Tris-HCl, pH6.8	3.31 ml
SDS	2 g
Glycerol	9 ml
2- mercaptoethanol	5 ml
1 % bromophenol blue	1 ml
ddH_20	80 ml

2.3.10 Site Directed Mutagenesis Buffers

2.3.10.1 Phosphorylation Buffer (5 x)

Tris.HCl, pH 7.5	250 mM
$MgCl_2$	50 mM
DTT	25 mM
Spermidine	0.5 mM
ATP	5 mM

2.3.10.2 Annealing Buffer (10 x)

Tris.HCl, pH 7.5	200 mM
$MgCl_2$	100 mM
NaCl	500 mM

2.3.10.3 Synthesis Buffer (5 x)

Tris.HCl, pH 7.5	50 mM
dNTPs	2.5 mM each
ATP	5 mM
DTT	10 mM

2.3.11 SSC (20 x)

Per litre:

NaCl	175.3 g
Sodium Citrate	88.2 g

Adjusted to pH 7.2. Sterilised by autoclaving

2.3.12 TAE (50 x)

Tris-acetate	2 M
EDTA, pH 8.0	50 mM

2.3.13 TBE (10 x)

Per litre:

Tris base	54 g
Boric acid	27 g
0.5M EDTA, pH8.0	20 ml

2.3.14 TE buffer (10 x)

Tris.HCl	10 mM
EDTA	1 mM

2.3.15 TEG

Tris.HCl, pH8.0	25 mM
EDTA	10 mM
Glucose	50 mM

2.3.16 Transformation buffer (TB)

PIPES	10 mM
$MnCl_2$	55 mM
$CaCl_2$	15 mM
KCl	250 mM

2.3.17 Tris-glycine Electrophoresis buffer (5 x) (For Laemmli SDS-PAGE)

Per litre:

Tris base	15.1 g
Glycine	94 g
10 % SDS	50 ml

2.3.18 Tris-Tricine Electrophoresis Buffers

2.3.18.1 Anode buffer

Tris.HCl, pH8.9	100 mM

2.3.18.2 Cathode Buffer

Tris.HCl, pH8.9	100 mM
Tricine	100 mM
SDS	0.1 %

2.3.19 Tris-Tricine PAGE

Formulations for Tris-Tricine separating, spacer and stacking gels:

	Separating 16 %	**Spacer 10 %**	**Stacking 4 %**
Acrylamide (40 %)	6 ml	1.5 ml	1.25 ml
Gel buffer	5 ml	2 ml	3.1 ml
Glycerol (80 %)	2.5 ml	-	-
APS	75 µl	30 µl	100 µl
TEMED	7.5 µl	3 µl	10 µl
ddH_2O	1.5 ml	2.5 ml	8.15 ml

APS was made fresh and added with TEMED immediately before pouring.

2.4 Suppliers

2.4.1 Enzymes

2.4.1.1 Restriction endonucleases

Restriction endonucleases were obtained from Gibco-BRL Life Technologies, Promega, New England Biolabs, Amersham Pharmacia Biotech, Boehringer Mannheim or Stratagene®.

2.4.1.2 Thermostable polymerase

Thermostable DNA polymerase for Polymerase Chain Reaction was obtained from Advanced Biotechnologies (Red Hot DNA Polymerase), Promega (*taq* polymerase), NBL Gene Sciences (Tbr DNA polymerase), or Boehringer Mannheim (Expand HiFi polymerase).

2.4.1.3 T4 DNA Ligase

T4 DNA ligase was obtained from Promega, New England Biolabs, or Boehringer Mannheim.

2.4.1.4 Klenow enzyme

Labelling grade DNA polymerase I, large fragment from *Escherichia coli*, was obtained from Boehringer Mannheim.

2.4.1.5 Pancreatic Ribonuclease A

Pancreatic RNase A was obtained from Sigma, and boiled for 10 minutes to remove any DNase activity before use as per manufacturer's instructions. Final concentration (10 mg/ml).

2.4.2.6 Alkaline phosphatase

Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

2.4.1.7 Sequenase®

Sequenase® was obtained from Amersham Pharmacia Biotech.

2.4.1.8 T4 Polynucleotide Kinase

T4 Polynucleotide Kinase was obtained from New England Biolabs.

2.4.1.9 T4 DNA Polymerase

T4 DNA Polymerase was obtained from New England Biolabs.

2.4.2 Isotopes

α -[^{32}P]dCTP and α -[^{35}S]dATP were obtained from Amersham Pharmacia Biotech or ICN. γ -[^{32}P]dCTP was obtained from Amersham Pharmacia Biotech.

2.4.3 General laboratory chemicals

All general laboratory chemicals were obtained from Sigma chemical company, Poole, Dorset or BDH, Poole, Dorset.

2.5 Bacteriological Methodology

2.5.1 Preparation of competent cells

Ultracompetent cells were prepared by the method of Inoue *et al.* (1990). Briefly, 200 ml of LB inoculated with the appropriate *Escherichia coli* strain were incubated at 20-23 °C in a 1 l Erlenmeyer flask on a rotating platform. Once the OD_{600nm} reached approximately 0.6, the cells were chilled on ice for 10 minutes, then harvested by centrifugation at 4250 rpm for 10 minutes. A second 10 minute incubation followed resuspension of the cells in 80 ml of ice cold transformation buffer (TB). The cells were then harvested and resuspended in 20 ml of TB. DMSO was added to a final concentration of 7% and the cells incubated for a further 10 minutes on ice before being aliquoted and snap frozen in liquid nitrogen.
Competent cells were stored at -80°C.

2.5.2 Transformation of competent cells

The DNA of interest was mixed with 200 µl of competent cells which had been allowed to thaw on ice. After incubation on ice for 30 minutes the mixture was heat shocked for 1 minute at 42 °C. Cells were returned to ice for a further 5 minutes then 800 µl of SOC medium added to the mixture and the cells incubated for 1 hour at 37°C. 150 µl of transformed cells were then spread on the appropriately supplemented agar plate and incubated overnight at 37°C.

2.5.3 Blue / white screening

Blue / white screening is used to differentiate recombinant from non-recombinant vectors in cloning experiments using vectors pTZ18r, pTZ19r, or Bluescript vectors which carry the lacZα gene and express acquired drug resistance(s). Briefly, 40 µl of a 50 µg/ ml solution of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethyl formamide and 4 µl of a 500 µg/ ml IPTG (isopropylthio-β-D-galactoside) in dimethyl formamide were spread on to the surface of the agar plate prior to spreading cells.

2.5.4 Bacterial cell mating

For introducing DNA into cells by mating the filter method was used. Briefly, approximately 10^7 donor cells and 10^8 recipient cells were mixed in a microcentrifuge tube, spotted onto a sterile 1 cm diameter nitrocellulose filter on an LB agar plate, and incubated at 23°C overnight. The filter was then vortexed in 1 ml of LB and the cells pelleted by centrifugation. The cell pellet was then resuspended in 100 µl LB and spread on agar plates supplemented with the appropriate antibiotics.

2.6 Nucleic Acid Methodology

2.6.1 Isolation of DNA

2.6.1.1 Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted following the method of Méjean *et al.* (1994). Two millilitres of an exponentially growing culture of bacterial cells were harvested by centrifugation and then resuspended in 400 µl of 10 mM EDTA pH 8.0. The cells were then lysed by the addition of SDS to a final concentration of 0.2 %. The lysate was extracted with an equal volume of phenol and centrifuged for 10 minutes at room temperature. The aqueous phase was then re-extracted with phenol:chloroform:isoamylalcohol (25:24:1), followed by a final extraction with chloroform. RNA was removed by incubation of the aqueous phase with 1/20 - 1/50 volume RNase ((10 mg/ml) at 37°C for 1 hour. Genomic DNA was then precipitated at -20°C by the addition of 0.3M sodium acetate and 3 volumes of ethanol. Following centrifugation at 4°C the DNA pellet was air dried, then resuspended in 400 µl ddH_20, and cleaned using a Promega Wizard DNA purification system, which is essentially an ion exchange resin.

2.6.1.2 Small scale plasmid DNA preparation

Cells (5 - 10 ml) were cultured overnight in the appropriate media and harvested by centrifugation at 5000 x g for 5 minutes. The pellet was resuspended in 150 µl TEG and transferred to a microcentrifuge tube. The cells were then lysed by the addition of 150 µl of alkaline lysis buffer and incubated on ice for 5 minutes. The tube was incubated for a further 5 minutes on ice following the addition of 150 µl 3 M sodium acetate and centrifuged (13,000 x g) for 15 minutes at 4°C. RNA was removed by incubation of the supernatant with 1/20 - 1/50 volume RNase at 37 °C for 30 minutes. The plasmid DNA was precipitated by the addition of 2 volumes of ethanol and incubation at –20 °C for 1 hour. The precipitate was collected by centrifugation (13,000 x g) at 4 °C for 15 minutes. The pellet was then air dried and resuspended in 30 µl ddH_20.

2.6.1.3 Caesium chloride plasmid preparation

Five hundred millilitres of overnight culture of the appropriate strain were harvested by centrifugation and the pellet resuspended in 4.5 ml of TEG and transferred to a sterile 30 ml Corex tube. 15 ml of alkaline lysis solution were added to the tube which was then inverted 3-4 times and incubated on ice for 5 minutes. Next, 11.25 ml of sodium acetate, pH 5.2, were added, the tube was inverted 3-4 times and incubated on ice for a further 40 minutes. The tube was then centrifuged at 10,000 x g for 40 minutes at 4 °C. The supernatant was transferred to two 30 ml Corex tubes and precipitated by the addition of 1 volume of propan-2-ol and incubation at –20 °C for 1 hour. The precipitate was collected by centrifugation at 10, 000 x g for 40 minutes. The resulting pellet was dissolved in 3 ml of 1 x TE. 3.8 g caesium chloride and 150 µl of ethidium bromide (10 mg/ml) were then added. The resulting solution was heat sealed in a 2 ml quick seal tube and centrifuged for 16 hours in a Beckman TL-100.2 rotor at 80,000 x g at 18 °C.

Following centrifugation the plasmid DNA was removed from the gradient by the use of a hypodermic syringe and needles. The ethidium bromide was removed by extraction with propan-2-ol saturated with NaCl and the DNA precipitated by incubation at –20 °C for 1 hour in 8 ml of 1 x TE and 8 ml of propan-2-ol. DNA was pelleted and collected as before and the pellet dissolved in 400 µl 1 x TE. Sodium acetate was added to 0.3 M and 2 volumes of ethanol added before incubating at –20 °C for 1 hour. The purified DNA was collected, desiccated and resuspended in an appropriate volume of double distilled H_2O.

2.6.1.4 Single stranded DNA preparation

To prepare single stranded DNA, the fragment of interest was first transferred to a phagemid vector which had the *f*1 origin of replication (pTZ18r, pTZ19r, or Bluescript vectors). A mid-log phase culture of the bacterium harbouring the vector of interest was infected with the helper phage M13 KO7 at a multiplicity of infection of ~1 and incubated at 37 °C for 1 hour. 400 µl of this culture were then transferred

to a 250 ml Erlenmyer flask containing 10 ml of LB with 70 μg/ml kanamycin, 100 μg/ml ampicillin and incubated at 37 °C overnight with vigorous aeration.
The overnight culture was centrifuged at 5000 x g for 15 minutes and the supernatant transferred to 5 x 1.5 ml microcentrifuge tubes. The phage were precipitated by the addition of 300 μl of 2.5 M NaCl/ 20 % PEG 8000. The solution was then mixed and incubated at room temperature for 15 minutes. Following centrifugation the pellets of all 5 tubes were pooled into one tube in 150 μl of 1 x TE and extracted with 50 μl phenol, and then with 500 μl chloroform. 0.1 volumes of sodium acetate pH 5.2 were added and the DNA precipitated by the addition of 3 volumes of ethanol and incubation at –20 °C for 1 hour. The DNA was collected, desiccated and resuspended in 30 μl ddH_2O.
For preparation of uracil-enriched DNA for site directed mutagenesis the host strain BW313 was used.

2.6.2 Polymerase Chain Reaction (PCR)

PCRs were typically performed in 50 μl volumes in 0.5 ml microcentrifuge tubes using a programmable Techne PHC-2 thermocycler. Reaction conditions were as follows:

DNA	80 - 100 ng
Forward primer	10 pmol
Reverse primer	10 pmol
dNTPs	200 μM
Polymerase	1 Unit
Reaction Buffer	1 x

Cycles were performed as follows:

1 x	denaturation	94 °C	5 minutes
35 x	denaturation	94 °C	30 seconds
	annealing	42 – 55 °C	30 - 90 seconds
	chain extension	72 °C	1 minute
1 x	chain extension	72 °C	10 minutes

2.6.3 Cleavage of DNA with restriction endonucleases

All restriction enzyme digestions were performed using enzymes from Gibco-BRL Life Technologies, Promega, New England Biolabs, Amersham Pharmacia Biotech, Boehringer Mannheim or Stratagene®. Buffers were used according to the manufacturer's specifications. For double digests involving different recommended buffers, the buffers were checked individually in double digests to determine which gave the most efficient digestion. All digests were carried out for 1 - 8 hours at the appropriate temperature.

2.6.4 Agarose Gel Electrophoresis of DNA

DNA was separated in 0.8 - 2.5 % (w/v) agarose (BRL electrophoresis grade) with 0.5 μg/ml ethidium bromide in 1 x TAE or 1 x TBE. Prior to loading, DNA samples were mixed with 1/5 volume of loading buffer. Electrophoresis was carried out horizontally across a potential difference of 1 - 10 V/cm. Gibco BRL 1 kb ladder was used for size markers. DNA was visualised by UV illumination and photographed.

2.6.5 Recovery of DNA from agarose gels

DNA fragments of interest were "cleaned" using a Gene Clean III (Bio 101) kit or QIAEX III Gel Extraction Kit, according to the manufacturer's instructions. Essentially three volumes of 6 M NaI were added to the gel slice which contained the DNA. Heating to 50 °C caused melting of the agarose to which 5 μl of glass milk were added and the incubation continued at 50 °C for 5 minutes. The silica-DNA

complex was then washed with salt-ethanol solution three times and the DNA eluted from the silica at 50 °C into MilliQ™ water.

2.6.6 Ligation of DNA fragments

Ligations were typically performed in 15 µl reaction volumes incubated overnight at 16 °C. Approximately 0.3 Units of ligase were used per reaction with 1 x ligation buffer supplied by the manufacturer. 1 mM hexaminecobalt chloride was also added to each reaction.

2.6.7 Dephosphorylation

To prevent self ligation of single cut vector DNA, calf intestinal alkaline phosphatase (CIAP) was used. Ligation of vector DNA can only occur when a DNA fragment with an intact 5' phosphate group is inserted. 0.01 Unit of CIAP was used to remove the 5' phosphate group of 1 pmol of linearised vector DNA as follows: After digestion of DNA with the appropriate restriction enzyme, CIAP was added directly to the digested mixture along with the supplied buffer. The mixture was then incubated at 37 °C for 30 minutes and then CIAP inactivated by heating to 65 °C for 5 minutes. Phenol/chloroform extraction was carried out to remove protein material and after centrifugation the aqueous phase was removed to a clean eppendorf tube. The DNA was precipitated by adding 0.3 M sodium acetate and ethanol and incubation at - 20 °C for 1 hour. DNA was pelleted by centrifugation and resuspended in an appropriate volume of ddH_2O.

2.6.8 Blunt ending of DNA fragments

To flush-end DNA molecules (5' overhang fill-in or 3' overhang removal) for ligation reactions, the DNA was precipitated from the digestion mix then added to the following: 3U T4 DNA polymerase, 100 µM each dNTP, 1 x T4 DNA Polymerase buffer (supplied by the manufacturer), and 50 µg/ml BSA. The mixture was incubated at 12 °C for 20 minutes followed by 75 °C for 10 minutes to inactivate the polymerase. Blunt ended DNA was then purified using Gene Clean™.

2.6.9 Site directed mutagenesis

2.6.9.1 Phosphorylation of Oligonucleotides

To 100 pmol of oligonucleotide, 5 µl of 5 x phosphorylation buffer, 5 Units of T4 polynucleotide kinase and sterile ddH_2O to a final volume of 25 µl were added. This mixture was then incubated at 37 °C for 40 minutes, the kinase was then inactivated by heating to 70 °C for 10 minutes. If the phosphorylated oligonucleotide was not used immediately it was stored at -20 °C until required.

2.6.9.2 Annealing of phosphorylated oligonucleotide to template DNA

1.25 pmol of each primer to be annealed were combined with 0.05 pmol of single stranded uracil-rich DNA in 1 x annealing buffer, in a total volume of 20 µl. The reaction was then heated to 70 °C for 5 minutes and allowed to cool over 35 minutes to 35 °C, then placed on ice.

2.6.9.3 Synthesis of complementary strand

To each annealing reaction the following were added: 6 µl 5 x synthesis buffer, 3 µl T4 DNA polymerase (10 U/µl), 0.7 µl T4 DNA ligase (2 U/µl) and sterile ddH_2O to a final volume of 30 µl. This mixture was then incubated at room temperature for 2 minutes before transfer to 37 °C for 2 hours.
Aliquots of the reaction mixture were transformed into ultracompetent *Escherichia coli* TG1 and BW313, and plated onto LB agar plates supplemented with ampicillin.

2.6.10 Isolation of RNA

2.6.10.1 Creating a Ribonuclease-free Environment

Before extraction of RNA, all equipment and surfaces were made free of contaminating ribonucleases: All surfaces were treated with RNAseZAP™ (Ambion) according to the manufacturer's instructions. Solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) overnight at 37°C, then autoclaved, with the exception of TBE which was made with DEPC-treated water. Electrophoresis tanks were soaked in DECON for 30 minutes, followed by a 0.5 % H_2O_2 solution for 30 minutes, then rinsed in DEPC-treated water.
Glassware was soaked in DEPC-treated water, autoclaved, then baked at 80°C for 2 hours before use.

2.6.10.2 Total RNA extraction

Total RNA was extracted from both aerobically and anaerobically growing cells of *S. frigidimarina* using the QIAGEN™ RNA/DNA Miniprep. kit as per the manufacturer's instructions. Typically, 35 μg of total RNA were extracted from 2 ml of exponentially growing cells. RNA was stored at -80 ° C.

2.6.10.3 Agarose Gel Electrophoresis of RNA

RNA was separated in 1.2% (w/v) agarose (BRL electrophoresis grade) with 0.5 μg/ml ethidium bromide in 1 x TBE. Prior to loading, RNA (1-10 μg) samples were mixed with 2 x Denaturing Sample Buffer (NEB) then heated at 75 °C for 5 minutes before snap-cooling on ice and loading. Electrophoresis was carried out horizontally across a potential difference of 2.5 V/cm. NEB RNA Ladder was used for size markers. RNA was visualised by UV illumination and photographed.

2.7 Detection of Homologous DNA fragments

2.7.1 Southern transfer

In order to detect specific sequences in DNA, it was separated in an agarose gel and transferred to a HYBOND-N™ nylon membrane before hybridisation with a DNA probe as follows:

After electrophoresis the gel was trimmed of extraneous parts and washed in two changes of denaturing buffer (500 ml) for 45 minutes. The gel was rinsed in 100 ml dH_20, then washed for a further 45 minutes in two changes of neutralising buffer (500 ml). After a second rinse in 100 ml dH_2O, the DNA was capillary-blotted out of the back of the gel onto the nylon membrane with ~ 1 litre 20 x SSC and UV crosslinked on to the membrane using a Stratagene™ UV Strata linker 1800.

For RNA, gels were blotted in 1 l of 1 x TBE immediately after electrophoresis.

2.7.2 Synthesis of Nick translated probes

The Promega nick translation system was used for incorporation of labelled dCTP into duplex DNA. Nucleotide mix (contains equal volumes of dATP, dGTP and dTTP), nick translation buffer, α–[^{32}P]dCTP and nick translation enzyme mix were mixed with 1 μg sample DNA in a total reaction volume of 50 μl. The mixture was incubated at 15 °C for 60 minutes, then 5 μl nick translation stop mix added. The probe was denatured by boiling for 5 minutes and snap-cooled on ice before use.

To check for incorporation of radioactivity, 5 μl of a 250-fold dilution were spotted onto two different glass fibre discs, and dried. One of the discs was then washed three times with ice-cold 10 % TCA, and a final wash with ethanol. Once the filter had dried, the fraction of radioactivity incorporated into acid precipitable material was checked by Geiger counting.

2.7.3 DNA blot hybridisation

Southern blots were prehybridised in 25 ml of prehybridisation solution for 1 hour at 42 °C in a rotating rotisserie. Denatured labelled probe was then added and the membrane hybridised for a further 12 hours. The membrane was then washed in 2 x SSC, 0.1 % SDS at room temperature for 10 minutes, followed by 1 x SSC, 0.1 % SDS at 42 °C for 10 minutes. For stringent washes this solution was replaced by 0.1 x SSC, 0.1 % SDS and incubated at 65 °C for 10 minutes. The membrane was then dried and exposed to X-ray film at - 80 °C for the required length of time.

2.7.4 RNA blot hybridisation

RNA blot hybridisation was carried out following the method of Kevil *et al.* (1997). Northern blots were prehybridised at 55 °C for 40 minutes in 50 ml of 0.25 M Na_2HPO_4 (pH 7.2); 7%SDS. Denatured labelled probe (section 2.7.2) was then added and the membrane hybridised for a further 8 hours. The membrane was then washed in 2 x SSC; 0.1% SDS, followed by 0.5 x SSC; 0.1% SDS, and finally 0.1 x SSC; 0.1% SDS, all at room temperature for 15 minutes. The membrane was then dried and exposed to X-ray film at - 80°C for the required length of time.

2.7.5 Construction of a library of *Shewanella frigidimarina* DNA

To construct a library of genomic DNA fragments, fragments of the size of interest were purified from a digest of genomic DNA by agarose electrophoresis. After ligation into the appropriate vector, aliquots of the ligation mix were transformed into ultracompetent *Escherichia coli* cells. Following overnight growth the cells were transferred to HYBOND-N™ nylon membrane by placing a piece of circular membrane onto the plate of colonies. After 30 - 60 seconds the membrane was removed and the DNA denatured by placing on filter paper soaked in denaturing solution for 3 minutes. The membranes were then neutralised by placing onto filter paper soaked in neutralising solution for 7 minutes. DNA was fixed to the membrane by UV crosslinking as described above.
Detection of positive colonies was as per section 2.7.3.

2.8 DNA sequencing

2.8.1 Automated sequencing

Samples for automated sequencing contained the following:

Dye Mix *	8.0 µl
Template:	
ssDNA	0.05 - 0.1 µg
dsDNA	0.3 - 0.5 µg
PCR product	30 - 180 µg
Primer	3.2 pmol
ddH_2O	to 20 µl

* Dye mix: ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit.

Sequencing cycles were performed as follows, on a Techne PHC 2 thermal cycler:

25 x	96 °C	30 seconds
	50 °C	15 seconds
	60 °C	4 minutes

DNA was precipitated from the reaction mixture as follows:
The reaction mixture was transferred to a 0.5 ml microcentrifuge tube containing 2.0 µl 3M sodium acetate (pH 5.2) and 50 µl ethanol, vortexed briefly then incubated on ice for 10 minutes. After centrifugation for 15 minutes the pellet was rinsed with 500 µl of 70 % ethanol, and dried. Samples not used immediately were stored at -80 °C.

Samples were separated on an ABI377 instrument according to the manufacturer's instructions. Data were collected using the ABI Prism™ program for the MacIntosh.

2.8.2 Manual sequencing

Manual sequencing of DNA was carried out using the Sequenase™ Version 2.0 kit (Amersham Pharmacia Biotech) which uses the dideoxy chain termination method. For each sequencing reaction (single stranded), the specific oligonucleotide primer (1 pmol) was annealed to its complementary DNA (approximately 1 μg DNA) in 1 x reaction buffer, by heating to 65 °C for 2 minutes and slowly cooling to room temperature.

To sequence double stranded DNA, the DNA was first denatured by the addition of 1 volume of a solution of 0.4 M NaOH/ 0.2 mM EDTA and incubation at 37 °C for 25 minutes. The DNA was then precipitated from 0.3 M sodium acetate, by the addition of 3 volumes of ethanol and incubation at - 20 °C for 1 hour. The DNA was collected by centrifugation, washed once with 70 % ethanol, and air dried. After resuspension in 7 μl sterile ddH_2O, 2.0 μl of Sequenase™ reaction buffer (5 x) and 1 μl specific oligonucleotide primer were added. The primer was annealed to the template as above. The reaction was placed on ice. Subsequent procedures for single stranded and double stranded sequencing were identical:

Extension from the annealed primer was achieved by addition of 1 μl 0.1 M DTT, 2 μl dNTP labelling mix (a 1 in 4 dilution of 7.5 μM dGTP, dCTP, dATP and dTTP), 0.5 μl [α^{35}S]dATP and 0.5 Units Sequenase™. The extension mixture was left at room temperature for 2 - 5 minutes. Further extension and termination was achieved by dispensing 3.5 μl of extension mix into 4 tubes preheated to 37 °C containing 2.5 μl of one of the four termination mixes:

ddATP mix	80 μM dNTPs; 8 μM ddATP; 50 mM NaCl
ddCTP mix	80 μM dNTPs; 8 μM ddCTP; 50 mM NaCl
ddGTP mix	80 μM dNTPs; 8 μM ddGTP; 50 mM NaCl
ddTTP mix	80 μM dNTPs; 8 μM ddTTP; 50 mM NaCl

The termination reaction was allowed to proceed at 37 °C for 5 minutes and the reaction stopped by the addition of 4 μl of stop solution.

Extension products were separated by electrophoresis through a 6 % denaturing polyacrylamide gel.

Sequencing reactions were heat denatured at 75 °C for 2 minutes before loading onto the gel and electrophoresed in a 1 x TBE buffer at 60 Watts for 1 - 6 hours.

The gel was transferred from the sequencing plates to blotting paper before fixing in 10 % (v/v) acetic acid and 10 % (v/v) methanol, and dried under vacuum at 80 °C for 1 - 2 hours. The gel was autoradiographed at room temperature overnight.

2.8.3 Sequence analysis

DNA sequence data were analysed using the UWGCG (University of Wisconsin Genetics Computer Group) programs (Devereux *et al*, 1994) on the unix-based mainframe HOLYROOD at Edinburgh.

2.9 SDS- Polyacrylamide Gel Electrophoresis of proteins

2.9.1 One dimensional SDS PAGE

This technique was used to separate proteins according to their molecular weight (Laemmli, 1970). The polyacrylamide gel was prepared in two phases, a resolving gel for the separation of protein samples and a stacking gel for the concentration of the protein samples before separation. The resolving gel was mixed well, poured between two glass plates and overlaid with dH_2O, then left to polymerise for 1 hour. Once the gel had polymerised, the water was removed and the stacking gel prepared. The stacking gel was mixed well then poured on top of the resolving gel. A comb was inserted into the top of the stacking gel and the gel allowed to polymerise for 30 minutes. Once polymerised the gel was clamped into a vertical electrophoresis tank filled with 1 x running buffer. The comb was then removed and the protein samples loaded into the wells.

Cell extracts were prepared as follows: 2 ml cell culture was harvested by centrifugation then resuspended in 100 µl 10 mM Tris-HCl pH 8.0. Samples were then heated to 90 °C for 10 mins and centrifuged. The supernatant was then removed to a fresh Eppendorf vial and boiled in 1 x SDS sample buffer, 1 x DTT for 10 minutes before loading. Such samples are referred to as "soluble fractions". All other protein samples were boiled in 1 x SDS sample buffer, 1 x DTT for 10 minutes before loading.

The gel was run at 10 volts per cm for 4-5 hours then stained with 1 % PAGE Blue Electran in 20 % (v/v) methanol, 5 % (v/v) acetic acid.

New England Biolabs Prestained wide range markers were used as size markers.

2.9.1.1 Tris- Tricine SDS PAGE

For high resolution separation of proteins Tris-Tricine gels were prepared. The method is essentially identical to the above, the differences arise in the composition of the gels (Schägger, 1994). Tris-Tricine gels are run at 4 °C overnight with 1 x anode buffer and 1 x cathode buffer at the appropriate electrode replacing the running buffer used for standard SDS PAGE.

2.9.2 Haem stain

In order to visualise *c*-type cytochromes the haem stain method of Thomas *et al.* (1976) was employed.

Following SDS PAGE, the gel was equilibrated for 5 minutes in 30 % methanol, 0.25 M sodium acetate (pH 5.2), then transferred to 30 % methanol, 0.25 M sodium acetate (pH 5.2) containing 400 μg/ml TMBZ. After incubation for 15 minutes in the dark, the stain was developed by the addition of 1 ml 30 % hydrogen peroxide. Once sufficient colour had developed the gel was fixed in 30 % methanol, 0.25 M sodium acetate (pH 5.2), and soaked in water before drying.

2.10 Assay for Fe(II) formation using ferrozine

Iron (III) dissimilation rates were assessed by monitoring formation of Fe(II) with the bidentate ligand ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine). The underlying principles and detailed methodology may be found in Dobbin *et al.*, 1995.

Briefly, 100 µl of culture medium were added to 1 ml of 10 mM ferrozine in a sterile Eppendorf and mixed by vortexing for 15 seconds. The mixture was then passed into a sterile cuvette containing 1 ml of 50 mM NaHEPES (pH7.0) through a 45 µm sterile filter. The absorbance was then measured at 562 nm. The cell density of each culture was also taken at A_{500nm} and A_{600nm}.

Anaerobic assays on whole cells were performed in 50 mM NaHEPES (pH 7.0) plus 350 µM ferrozine and initiated by the injection of 100 µM Fe(III). Additions of an electron donor (500 µM) were made 5 minutes before an assay.

Iron standards were made by serial dilution of 1000 µg/dL iron standard (SIGMA), in 5 % w/v hydroxylamine.HCl. Calibration plots are given in appendix III.

Chapter 3

Cytochrome *c*$_5$ from *Shewanella frigidimarina* NCIMB400: Cloning, sequencing, and expression

Investigation of the respiratory chain of *Shewanella frigidimarina* NCIMB400 has shown the periplasm to contain several *c*-type cytochromes, the most abundant in anaerobic conditions being flavocytochrome c_3 (Morris, 1987). It was during purification of this flavocytochrome that a reduced cytochrome was identified. This small cytochrome has been purified and characterised biochemically (F. Allen, unpublished work). Comparison of the N-terminal sequence with available protein sequences showed the protein to be similar to the class I cytochromes c_5 from other bacteria (Section 1.6.2).

Despite extensive biochemical and structural characterisation of cytochromes c_5 from *Azotobacter vinelandii* and *Pseudomonas mendocina* the biological function of the protein still appears to be subject to some speculation.

Information concerning the structure and function of proteins usually proceeds through such biochemical characterisation to amino acid sequencing. However, to understand fully both the evolution and role of a particular polypeptide it is necessary to further identify and isolate the cognate gene.

The long range goal of this work is to identify the physiological role and redox partners for cytochrome c_5 of *S. frigidimarina* NCIMB400. There is a tendency in bacterial genomes for proteins of related function to be cotranscribed in multicistronic operons. The initial aim, therefore, was to clone and sequence the structural gene for cytochrome c_5 and sequence the surrounding regions of genomic DNA to identify any proteins likely to be functionally related.

In this chapter, cloning and sequencing of the cytochrome c_5 gene and surrounding sequence is described. The sequence of the protein is compared with other cytochromes c_5. Expression of *S. frigidimarina* cytochrome c_5 is also examined, and results of overexpression of the protein in *E. coli* and *Shewanella* are given.

3.1 Cloning the structural gene encoding cytochrome c_5

3.1.1 PCR using degenerate primers

At the outset of this work, a partial amino acid sequence of cytochrome c_5 from *S. frigidimarina* NCIMB400 had been determined. On the basis of this sequence, three oligonucleotides were designed to act as primers in polymerase chain reactions (PCR). Owing to the redundancy of the genetic code, all oligonucleotides were designed as "best guesses" based on the known codon preferences (Appendix II): The forward primer, N3187, is 22 nucleotides in length with 64-fold degeneracy; P3928, a second forward primer, and N3188, the reverse primer, both have 256-fold degeneracy within 25 and 28 nucleotides respectively (for oligonucleotide sequences see section 2.1.4).

Primers additionally include, at the 5' end, an eight base extension containing a restriction endonuclease cleavage site. These exogenous sequences become incorporated into the PCR product and facilitate subsequent cloning of the product. The extensions also contribute to the reannealing of primers to PCR products generated after the initial PCR cycle.

Following empirical determination of the annealing temperature of the degenerate primers, a fragment of the expected 88 base pairs was obtained (Figure 3.1). Due to the sequence heterogeneity of the primers, a 10-fold increase in primer concentration was needed, in comparison with standard PCR reactions.

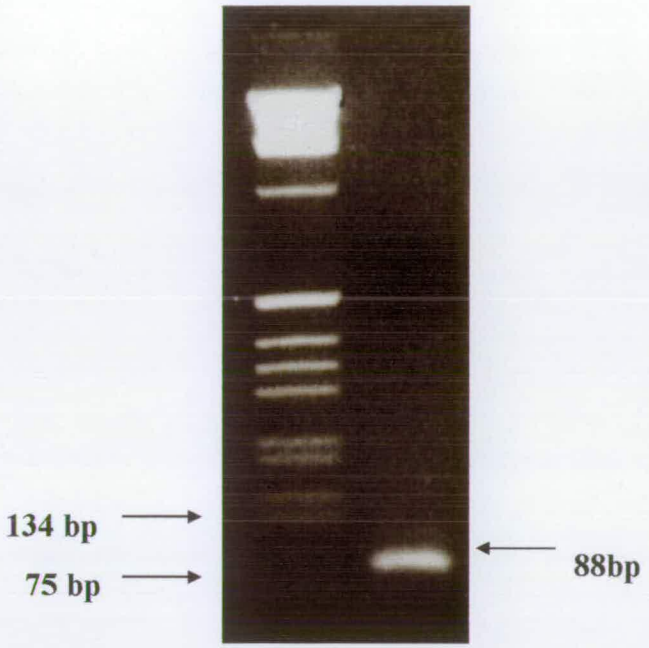


Figure 3.1 Agarose gel electrophoresis of PCR amplified products using primer pair N3187 and N3188; Reaction cycles as follows: 1 x 94°C, 3 mins.; 35 x 94°C, 35 s; 45°C, 45 s; 72°C, 1 min., and 1 x 72°C 10 mins.. Reactions were performed in a solution containing 100 ng genomic DNA, 11 pmol each primer, 200 μM dNTPs, and 1 Unit Expand HiFi DNA polymerase in 1 x reaction buffer. Products were separated through a 2.0 % agarose gel. Gibco BRL 1 kb ladder were used as size markers.

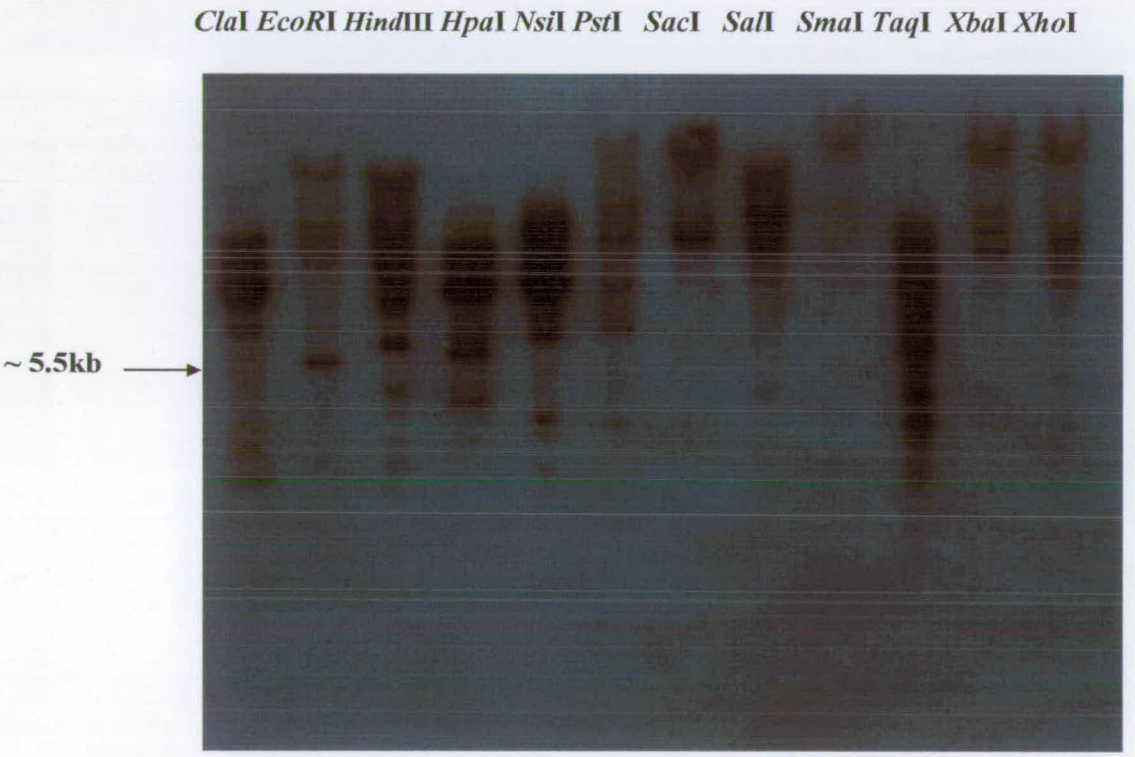
The 88 bp PCR product was sequenced directly and found to encode the predicted region of the cytochrome c_5 structural gene (results not shown).

3.1.2 Construction and screening of a *S. frigidimarina* genomic DNA library

Following ^{32}P labelling, the 88 bp PCR product was used for Southern blotting of genomic DNA digested with a variety of restriction endonucleases. A band of approximately 5500 bp was identified following digestion with *Eco*RI (Figure 3.2). A genomic library was then constructed from size-fractionated *Eco*RI fragments cloned into the *Eco*RI site of pTZ18r. Screening of over 10, 000 colonies with the labelled PCR product produced 12 putative positive colonies. Plasmid DNA was extracted from these

colonies and used in a third round of screening. A plasmid, pAH3, containing an approximately 5500 bp insert was identified which hybridised strongly to the labelled PCR product. This fragment, a total of 5634 bp, was then sequenced (primers 3x - 3x7, 3xr - 3xr2).

Figure 3.2 Autoradiograph of a Southern blot of *S. frigidimarina* genomic DNA probed with an α^{32}P-labelled PCR fragment corresponding to the N-terminal region of *S. frigidimarina* cytochrome c_5. The blot was washed in 2x SSC; 0.1 % SDS at room temperature for 15 mins, then at 65 °C for 15 mins before exposure.



The large sizes of fragments shown in figure 5.2 suggests that the genomic DNA has only been partially digested by the given restriction enzymes.

3.1.3 Sequencing of clone pAH3 and database comparisons

A search of the SWISSPROT database by the program FastA developed by the University of Wisconsin Genetic Computer Group (UWGCG; Pearson and Lipman, 1988) using the initial region of the clone pAH3 as the query sequence indicated similarity with sequences encoding cytochrome *b* involved in the eukaryotic

mitochondrial respiratory chain. Sequencing further revealed another region of DNA encoding a homologue of an iron-sulfur cluster protein, a product of the *nif* gene cluster of *Azotobacter vinelandii*. Sequencing of the entire fragment revealed the region to encode a protein with strong similarity to members of the HesB/ YADR/ YFHF family of proteins (Fleischmann *et al.*, 1995; Figure 3.3). This family of proteins is as yet uncharacterised, although it is thought that the HesB protein of *Azotobacter vinelandii* is involved in nitrogen fixation.

Figure 3.3 Results of sequence comparison of pAH3 with protein sequences available in the SWISSPROT and EMBL databases, using FASTA.

Sequence name	**Protein/organism**	**Accession number**	**Smallest sum probability**
YADR_HAEIN	Hypothetical protein/ *Haemophilus influenzae*	P45344	2.8e-55
YADR_ECOLI	Hypothetical/ *Escherichia coli*	P37026	1.0e-54
Y00F_MYCTU	Hypothetical/ *Mycobacterium tuberculosis*	Q10393	6.0e-27
YNIU_RHOSH	Hypothetical/ *Rhodobacter sp.*	Q01195	4.9e-22
e213904	unknown / *Rhizobium sp.*	Z68203	1.6e-20
142359	ORF 6 / *Azotobacter vinelandii*	M20568	4.0e-19
YNIU_RHOCA	Hypothetical / *Rhodobacter capsulatus*	Q07184	7.6e-19
2183309	HesB / *Cyanothece* PCC	AF0037000	1.2e-17
YNIS_BRAJA	Hypothetical / *Bradyrhizobium japonicum*	P37029	1.6e-16
HEB2_ANAVA	HesB protein / *Anabaena*	P46052	1.4e-15
YFHF_HAEIN	Hypothetical / *Haemophilus influenzae*	P44672	3.1e-15
497437	ORF 2 / *Frankia alni*	L29299	3.3e-15
d1019437	Hypothetical / *Synecoccus*	D90916	5.5e-15
HESB_PLEBO	HesB protein /	P46053	7.1e-15
HESB_ANASP	HesB protein / *Anabaena*	P18501	5.2e-14
2271523	*Isc*A/ *Azotobacter vinelandii*	AF010139	5.4e-14
847777	ATP-binding protein / *Azotobacter vinelandii*	U26427	6.8e-14
HEB1_ANAVA	HESB / *Anabaena*	P46051	9.0e-14

The G+C content of the sequenced region is calculated at 40.66 %, a value which agrees well with the value of 40-43 % expected for strains of *Shewanella frigidimarina* (Bowman *et al.*, 1997).

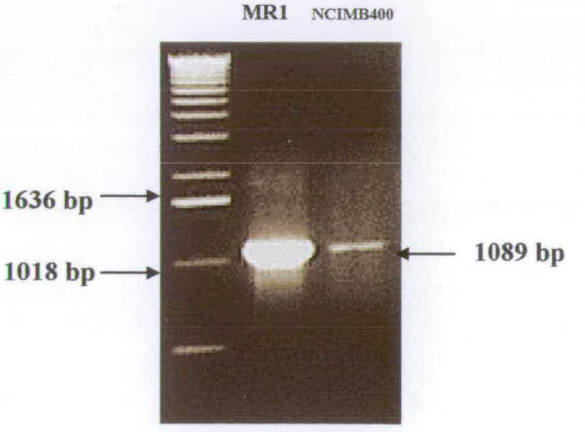
Analysis of the open reading frames identified from translation of the DNA sequence did not however reveal the structural gene for cytochrome c_5 in this clone, and a new approach had to be adopted.

3.1.4 PCR based on *S. putrefaciens* cytochrome c_5

Until very recently no DNA sequence information was available for any of the cytochromes c_5 identified in *Azotobacter*, *Pseudomonas*, or *Shewanella*. The first genetic sequence was published in 1997 by Rey and Maier, for *Azotobacter vinelandii*. Since then, the structural gene for *Shewanella putrefaciens* MR1 cytochrome c_5 has been isolated (Saffarini *et al.*, 1998). Following the publication of the cytochrome c_5 sequence from *S. putrefaciens* MR1, a pair of oligonucleotide primers were designed to amplify a 1089 bp region of *S. putrefaciens* genomic DNA encompassing the entire structural gene (MPF and MPR; section 2.1.4). Comparison of several proteins isolated from both *S. frigidimarina* NCIMB400 and *S. putrefaciens* has revealed significant sequence identity between homologues from these two *Shewanella* strains (Reid *et al.*, unpublished). The region of *S. putrefaciens* DNA amplified by PCR should, therefore, act as an efficient probe to screen *S. frigidimarina* genomic DNA for the cytochrome c_5 structural gene.

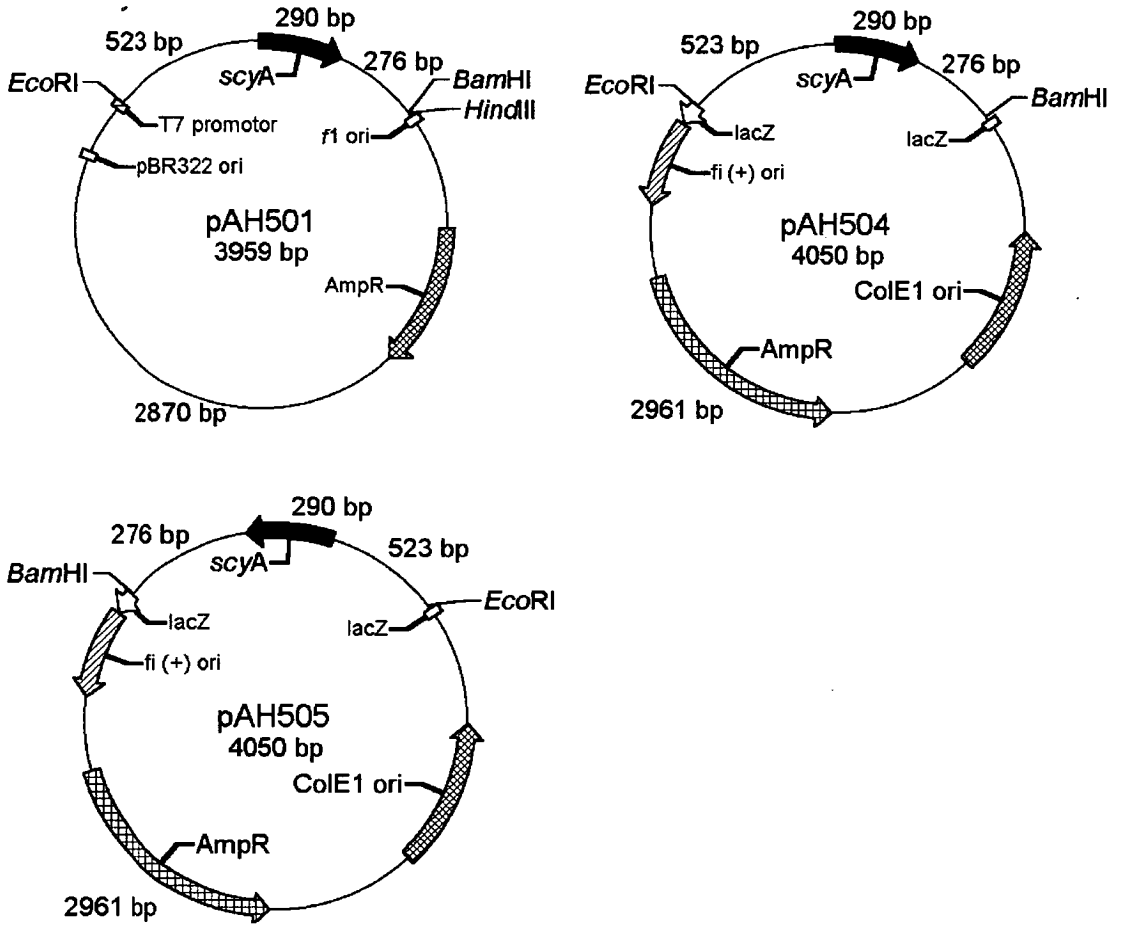
Following PCR, the expected product was obtained for *S. putrefaciens* MR1. In addition, PCR using *S. frigidimarina* NCIMB400 genomic DNA as the template, also resulted in a 1089 bp product (Figure 3.4).

Figure 3.4 Agarose gel electrophoresis of PCR amplified products using primer pair MPF and MPR; Reaction cycles as follows: 1 x 94°C, 3 mins.; 35 x 94°C, 35 s; 50°C, 45 s; 72°C, 1 min., and 1 x 72°C 10 mins.. Reactions were performed in a solution containing 100 ng genomic DNA, 10 pmol each primer, 200 μM dNTPs, and 1 Unit *Taq* DNA polymerase in 1 x reaction buffer. Products were separated in a 0.8 % agarose gel.



Taking advantage of the restriction endonuclease sites incorporated into the PCR primers, the 1089 bp fragment of *S. frigidimarina* DNA was cloned into pTZ18r and the Bluescript vectors, pKS$^-$ and pSK$^-$, on an *Eco*RI-*Bam*HI fragment, to give pAH501, pAH504, and pAH505, respectively (Figure 3.5). DNA sequencing confirmed that the fragment corresponded to the predicted region of *Shewanella* DNA.

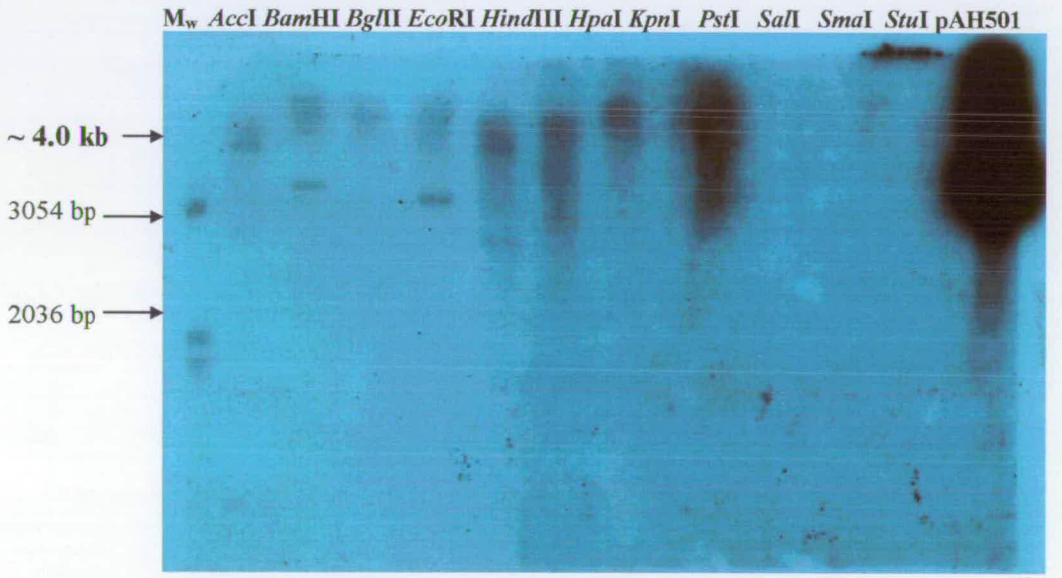
Figure 3.5 Plasmid maps of pAH501, pAH504, pAH505, constructed by cloning a 1089 bp region of PCR amplified *S. frigidimarina* DNA into the multipurpose vectors pTZ18r, pSK- and pKS-, respectively.



In order to obtain a fragment of *S. frigidimarina* DNA with a sufficient length of flanking DNA for sequence comparisons, clone pAH501 was used as follows for Southern blotting: Digestion of pAH501 with *Eco*RI and *Bam*HI released the PCR

product which was purified with QIAGEN QIAEX III and then labelled with [α^{32}P]dCTP by nick translation. This ^{32}P labelled probe was used to probe various restriction digests of *S. frigidimarina* genomic DNA on Southern blots. An approximately 4,000 bp *Acc*I fragment was identified which was predicted to contain the entire cytochrome c_5 structural gene and a significant length of flanking DNA (Figure 3.6).

Figure 3.6 Autoradiograph of a Southern blot of *S. frigidimarina* genomic DNA probed with a ^{32}P-labelled PCR fragment corresponding to the region of *S. frigidimarina* DNA encoding the structural gene for cytochrome c_5. The blot was washed in 2x SSC; 0.1 % SDS at room temperature for 15 mins, then at 65 °C for 15 mins before exposure. Plasmid pAH501 was included as a positive control.



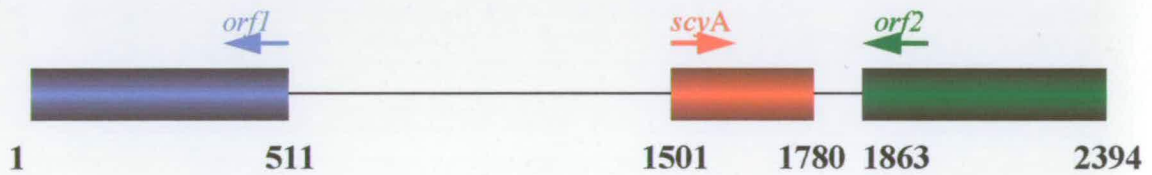
A genomic library was constructed from size-fractionated partially digested *Acc*I fragments cloned into the *Acc*I site of pTZ18r. Screening of approximately 4,000 colonies with the labelled 1089 bp PCR fragment produced 6 putative positive colonies. Plasmid DNA was extracted from these colonies and used as templates in PCR reactions using primers MPF and MPR, as above. Following agarose gel electrophoresis of the PCR products, one of the samples was found to contain the 1089 bp fragment expected with amplification using these primers. The plasmid DNA from this colony, named

pAH506, along with plasmids pAH501-505 (2.1.2), were used to sequence the entire region containing the cytochrome c_5 structural gene on both strands.

3.2 Sequence of *Shewanella frigidimarina* NCIMB400 cytochrome c_5

A total of 2394 bp from the ~ 4,000 bp insert of pAH506 was sequenced. The organisation of this region is shown in figure 3.7; the cytochrome c_5 structural gene is referred to as *scy*A in keeping with the convention of Saffarini *et al.* (1998).

Figure 3.7 Organisation of the region encoding cytochrome c_5. Numbers given below the figure correspond to the sequence shown in figure 3.8. Arrows indicate the direction of transcription; "orf" - *o*pen *r*eading *f*rame.



A combination of subcloning and designed primers were used to sequence this entire region on both strands (Figure 3.8). Oligonucleotide primers were designed on the basis of sequence from one strand alone as part of an efficient primer walking approach.

Figure 3. 8 The DNA sequence from clone pAH506. Amino acid sequence from *o*pen *r*eading *f*rame 1, *ccm*A, is highlighted in blue (3' – 5'), *orf* 2, *scy*A, in red (5' – 3'), and *orf*3, *nrf*G in green (3' – 5'). Arrows indicate direction of transcription.

```
 3′ tgagttgtctatgcggcggaaagcgtctggttgccaattacgatgcggttagcgtggatt
1   ---------+---------+---------+---------+---------+---------+
 5′ actcaacagatacgccgcctttcgcagaccaacggttaatgctacgccaatcgcacctaa
      S  L  L  V  G  G  K  R  L  G  V  T  L  A  V  G  I  A  G  L

    ctcactgttgtgacctcacggatttcggtcacaccgttagttccgaggtatcgacaaccg
61  ---------+---------+---------+---------+---------+---------+
    gagtgacaacactggagtgcctaaagccagtgtggcaatcaaggctccatagctgttggc
      L  S  L  V  P  T  G  L  A  L  T  A  I  L  A  G  Y  S  N  A

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      E  L  N  L  L  V  A  L  L  P  A  I  L  I  L  P  V  G  T  L

    attggtcactcgattatgaaaacggttatgattttaccgctcgccaacacctgattcgtc
181 ---------+---------+---------+---------+---------+---------+
    taaccagtgagctaatacttttgccaatactaaaatggcgagcggttgtggactaagcag
      L  W  H  A  L  V  K  A  L  V  L  I  A  L  P  Q  P  S  L  L

    gtagacgagattcgacggtagtgattttagccgaaatttttccgcgagttcgctatcgta
241 ---------+---------+---------+---------+---------+---------+
    catctgctctaagctgccatcactaaaatcggctttaaaaaggcgctcaagcgatagcat
      M  Q  E  L  S  G  D  S  F  D  A  K  F  L  R  E  L  S  L  M

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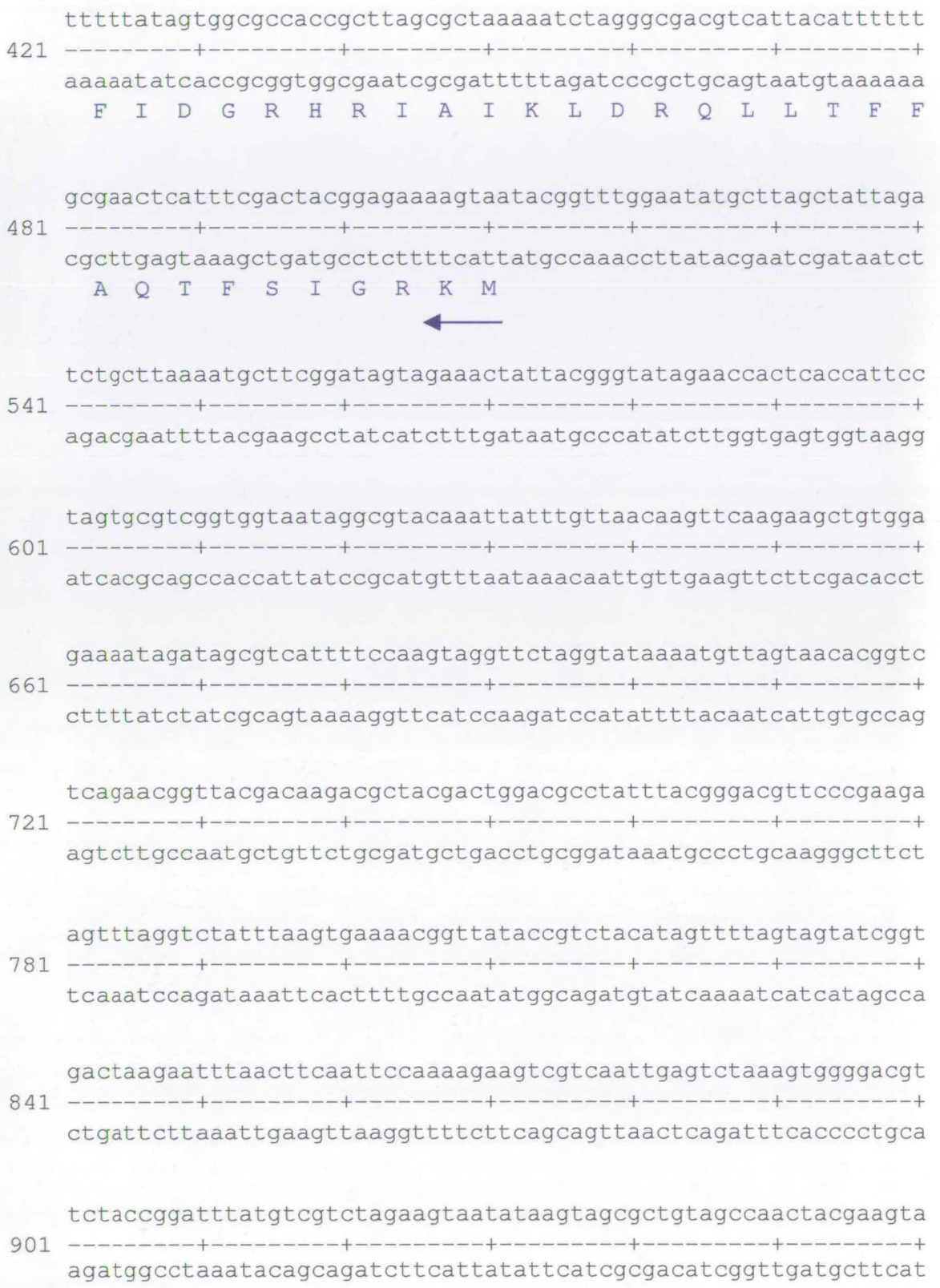
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agatggcctaaatacagcagatcttcattatattcatcgcgacatcggttgatgcttcat

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gcacaagatgctgaagctatttataataaggcatgtaccgtatgccatagcatgggtgtt
A Q D A E A I Y N K A C T V C H S M G V
cgaccacgaggatttagggtgttatgacgactgacccttggtgcaaatcgatttccacaa
1621---------+---------+---------+---------+---------+---------+
gctggtgctcctaaatcccacaatactgctgactgggaaccacgtttagctaaaggtgtt
A G A P K S H N T A D W E P R L A K G V
ctattagatcaattttcacatttttgacctaatttacgttacggtggtccaccatacaca
1681---------+---------+---------+---------+---------+---------+
gataatctagttaaaagtgtaaaaactggattaaatgcaatgccaccaggtggtatgtgt
D N L V K S V K T G L N A M P P G G M C
tgtctaacatggctacttctaatgtttcgatgctaactcaaatacagatttcgattcatt
1721---------+---------+---------+---------+---------+---------+
acagattgtaccgatgaagattacaaagctacgattgagtttatgtctaaagctaagtaa
T D C T D E D Y K A T I E F M S K A K *
aaaatgacatatttttttggccgtactcggccaaaaaaatatggaagcattacggttttc
1781---------+---------+---------+---------+---------+---------+
ttttactgtataaaaaaaaccggcatgagccggtttttttataccttcgtaatgccaaaag
tgaatcgcgataacatctactaaataacatgaactcataggacatgattcacgcgtcaac
1841---------+---------+---------+---------+---------+---------+
acttagcgctattgtagatgatttattgtacttgagtatcctgtactaagtgcgcagttg
* Q V Q T D L V L H A T
gccaaagtgggtgtatgtgaaacgattagaaaggaacctatagtggtcgaacaccaaact
1901---------+---------+---------+---------+---------+---------+
cggtttcacccacatacactttgctaatctttccttggatatcaccagcttgtggtttga
D T E G V Y V K S I K G Q I D G A Q P K

```
    ataacggtacgaatctattctggcgatactgaagttgtgatcgacaaaccgaattaaatt
1961--------+---------+---------+---------+---------+---------+
    tattgccatgcttagataagaccgctatgacttcaacactagctgtttggcttaatttaa
    I  N  G  H  K  S  L  V  A  I  V  E  V  S  A  T  Q  S  L  K

    gtagtgggggtctggtcacgatagtagattacagtggcaatgtccattcctaaaacgcg
2021--------+---------+---------+---------+---------+---------+
    catcacccccagaccagtgctatcatctaatgtcaccgttacaggtaaggattttgcgc
    V  D  G  G  V  G  T  S  D  D  L  T  V  T  V  P  L  S  K  A

    actggaaccagcgacgattgccatgaaatcccggaagtcaacgtgcccgcttatgtttct
2081--------+---------+---------+---------+---------+---------+
    tgaccttggtcgctgataacggtactttagggccttcagttgctcgggcgaatacaaaga
    S  V  K  T  A  A  L  P  V  K  P  G  E  T  A  R  A  F  V  F

    atcacagtagacctggtcgaaagcggcgttagagaccactctaactttagcgttatgagt
2141--------+---------+---------+---------+---------+---------+
    tagtgacatctggacctgctttcgccgcaatctctggtgagattgaaatcgcaatactca
    I  T  D  D  P  G  A  K  A  A  L  E  P  S  I  S  I  A  I  S

    gactaaatgaacactgaaaacggacgaacacatgtagtagcaatccgtacggtctccaaa
2201--------+---------+---------+---------+---------+---------+
    ctgatttacttgtgacttttgcctgcttgtgtacatcatcgttaggcatgccagaggttt
    V  S  K  S  T  V  K  A  Q  K  H  V  D  D  N  P  M  G  S  T

    gccgaacttatgcattcaatcgacaaagttagcgtaagtagtcacgtcgcgctagatgta
2261--------+---------+---------+---------+---------+---------+
    cggcttgaatacgtaagttagcagtttcaatcgcattcatcagtgcagcgcgatctacat
    E  A  Q  I  R  L  N  A  T  E  I  A  N  M  L  A  A  R  D  V

    gacccgccaatgacagctcttaccaaacggtacttaactatcgaaaaaccatgaatcgac
2321--------+---------+---------+---------+---------+---------+
    ctgggcggttactgtcgagaatggtttgccatgaattgatagcttttggtacttagctg
    D  P  R  N  S  D  L  I  T  Q  W  S  N  I  A  K  Q  Y  K  A

    Acttctttcgtagg  5′
2381--------+----     2394
    tgaagaaagcatcc  3′
    T  F  F  A  D
```

The 2394 bp region sequenced has a G+C content of 42.3 % which compares well with the value of 40-43 % determined from thermal denaturation profiles of several *S. frigidimarina* strains (Bowman *et al.*, 1997).

3.2.1 Sequence analysis

3.2.1.1 *scy*A, the *S. frigidimarina* NCIMB400 cytochrome c_5 structural gene

The coding region of the cytochrome c_5 structural gene, named *scy*A, was located by translating the DNA sequence in both directions, and searching for the presence of *o*pen *r*eading *f*rames (*orf*s) in all six reading frames.

The *orf* encoding cytochrome c_5 begins with an ATG start codon at position 1501, and is preceded by a putative Shine-Dalgarno sequence (5'-AGGA-3') at a distance of 8 nucleotides, which is the likely ribosome binding site for *scy*A (Figure 3.9). At a position of 71- 94 nucleotides upstream of this ATG codon sequences resembling the consensus for σ^{70}-dependent *E. coli* promoters may be found (Figure 3.9).

A second ATG start codon is located at position 1441 (Figure 3.9). However, it may be discounted because translation from this start point would result in an unusually long and atypical periplasmic signal sequence (see further), and also it is not adjacent to any possible ribosome binding site.

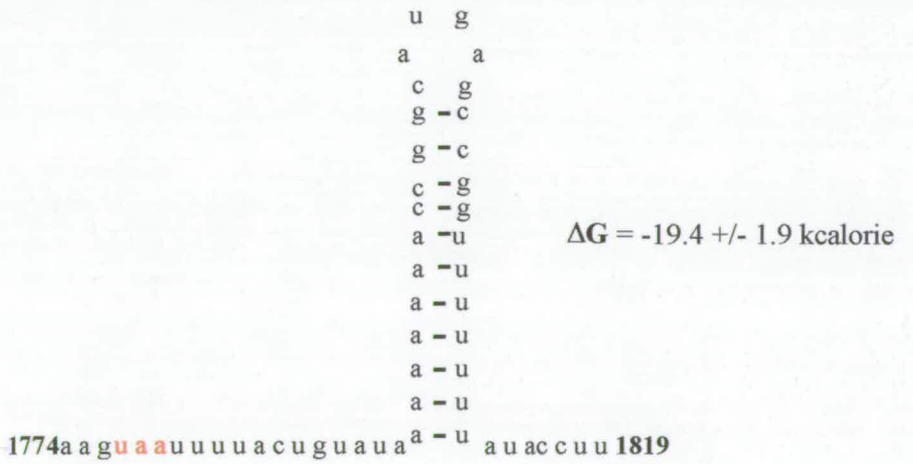
Figure 3.9 Details of the DNA sequence upstream of *scy*A. ATG start codon is shown in green, and the putative Shine-Dalgarno sequence in red. Possible -35 and -10 hexamers are indicated in blue (see text). Numbers correspond to those in figure 3.8.

```
          -35 hexamer                    -10 hexamer
1396  aaagatgaga tttattgaag tttgtactaa ttttagtcgc tataatgagt ctgcctcaaa

                                        SD
1456  gggcagcgag agctttgtac gtatttgcat taggaacatt gaacatgaaa aaactgttag
                                                  scyA ⟶
```

The *orf* stops with a TAA at position 1778 (Figure 3.10). A potential stem-and-loop transcription terminator is found 12 nucleotides downstream of this stop codon (Figure 3.10). Its presence indicates that transcription of *scy*A might use a *rho*-independent termination mechanism and also implies that cytochrome c_5 is not co-transcribed with any other sequences.

Figure 3.10 Details of the DNA sequence downstream of *scy*A. The free energy value of the stem-and-loop structure, calculated by the method of Tinoco *et al.* (1973), is shown. The stop codon of *scy*A is highlighted in red.



3.2.1.2 Other open reading frames

Initial inspection for the presence of open reading frames revealed that there are no open reading frames co-transcribed with that coding for cytochrome c_5 (*scy*A). The closest *orf*, at a distance 87 residues downstream of the *scy*A stop codon, encodes a homologue of *E. coli* NrfG (*orf*2; Figure 3.8). In *E. coli*, NrfG is encoded as part of an operon encoding *c*-type cytochromes necessary for *n*itrate *r*eduction by *f*ormate (Hussain *et al.*, 1994). NrfG, along with NrfE and NrfF, is thought to fulfil a highly specialised role in the assembly of *c*-type cytochromes (Grove *et al.*, 1996; Grove *et al.*, 1996a). In *S. frigidimarina* this operon is transcribed in the opposite orientation to cytochrome c_5 (Figure 3.7 and 3.8). Indeed, the two transcription units probably end at the same terminator, since it should be bidirectional.

The closest open reading frame upstream is located at a distance of 990 nucleotides from the cytochrome c_5 structural gene, and is also transcribed in the opposite orientation (*orf*1; Figure 3.8). Comparison of the protein sequence with those available in the SWISSPROT database reveal similarity to the *ccm*A gene product of *E. coli*. Mutation of the operon encoding CcmA in *E. coli* results in the absence of all of the known *c*-type cytochromes (Grove *et al.*, 1996a). The genes encoded in this operon are involved in *c*ytochrome *c* *m*aturation.

These open reading frames and the non-coding regions (nucleotides 511-1501 and 1780 - 1863) are homologous with the flanking regions of the cytochrome c_5 structural gene from *Shewanella putrefaciens* MR1.

3. 2. 1.3 Regulatory regions

The intergenic region between *ccm*A (*orf*1) and *scy*A was examined for possible regulatory sites. A putative EtrA box may be found at position 1272-1285, 237 bp upstream of the start codon. As shown in table 3.1, this EtrA box differs by 2 bases from

the *E. coli* FNR consensus sequence. In *E. coli,* however, the FNR box is usually located between 3 and 13 bases upstream of the -35 hexamer.

Table 3.1 Alignment of the putative EtrA box upstream of *scy*A with consensus FNR binding sites of other organisms.

scyA	TTGAT	N_4	TTCCA
E. coli	TTGAT	N_4	ATCAA
Paracoccus pantotrophus	TTGAC	N_4	CTCAA
Alcaligenes denitrificans	TTGAT	N_4	GTCAA
Pseudomonas aeruginosa	TTGAC	N_4	ATCAG
Alcaligenes faecalis	TTGAT	N_4	ATCAA

Two possible σ^N-dependent promotors have also been identified upstream of *scy*A. The most plausible starts near position 1255 (Table 3.2 A), and would give an mRNA strand of ~ 560 nucleotides (termination will be around 1816). The second weaker promotor starts near 1135, giving an mRNA strand of ~ 680 nucleotides (Table 3.2 B).

Table 3.2 Alignment of the putative σ^N-dependent promotors upstream of *scy*A with the consensus site from *E. coli*.

A	GGGGTT	N_5	TTGCT
B	CGGATG	N_5	CAGCT
Consensus	TGGCAC	N_5	TTGC$_{T/A}$

No sequences resembling any other consensus regulatory sites, such as NarL heptamers, could be found upstream of *scy*A.

3.3 Examination of the ScyA protein sequence

3.3.1 Cytochrome c_5 is localised to the periplasm

The inferred amino acid sequence of the *S. frigidimarina* ScyA corresponds to a protein sequence of 99 amino acids with a calculated molecular weight 10, 299 Da.
The predicted cytochrome c_5 structural gene product, however, contains a 21 amino acid N-terminal signal sequence which is not found in purified cytochrome c_5 (F.Allen, unpublished). This N-terminal sequence, shown in figure 3.31, conforms to the format of a typical prokaryotic signal peptide with positively charged N-terminal residues followed by a hydrophobic region, so it is predicted to be removed from the mature peptide. The cleavage site follows the -3,-1 rule which states that the residues at positions -3 and -1 relative to the cleavage site are small and neutral for cleavage to occur correctly (von Heijne, 1995). In this case, the residues at -3 and -1 are valine and alanine respectively (Figure 3.11).

Figure 3.11 Comparison of the NH_2-terminal signal peptide sequences for three periplasmic *c*-type cytochromes from *S. frigidimarina* NCIMB400. The signal peptidase cleavage site is marked by a vertical line , -3 and -1 residues are highlighted in red, and the first amino acid of the mature polypeptide in blue. Each signal sequence was checked using the signal peptide prediction program, Version 1.1 (Nielsen *et al.*, 1997).

		-3 -1	+1
Cytochrome c_5	MKKLLAMTAVAALTMSVNVS	A	Q
Cytochrome c_3	MSNKLLSALFAAGFAVMMMSSAS	F A	A
Flavocytochrome c_3	MKKMNLAVCIATLMGTAGLMGTAV	A	A

The mature cytochrome c_5 apoprotein should therefore be located in the periplasm with a molecular mass of 8298 Da. Cytochrome c_5 has one consensus sequence (C-X-X-C-H) for haem binding, and the holoprotein, with a single haem bound, has a calculated molecular weight of 8910.5 Da, a value lower than the molecular weight of ~10,000 Da as judged by SDS Polyacrylamide Gel electrophoresis.

The predicted localisation of cytochrome c_5 to the periplasm is in agreement with the finding of Morris (1987), who isolated the protein from the periplasm of *S. frigidimarina*. Chou-Fasman prediction analysis of the amino acid sequence, using the PLOTSTRUCTURE program from UWGCG, indicates that the protein is predominantly hydrophilic. There are no putative transmembrane regions. These results contrast with the work of Hunter *et al.* (1989) who supply evidence that *Pseudomonas stutzeri* and *Azotobacter vinelandii* cytochromes c_5 are predominantly membrane bound.

3.3.2 Amino acid sequence comparisons

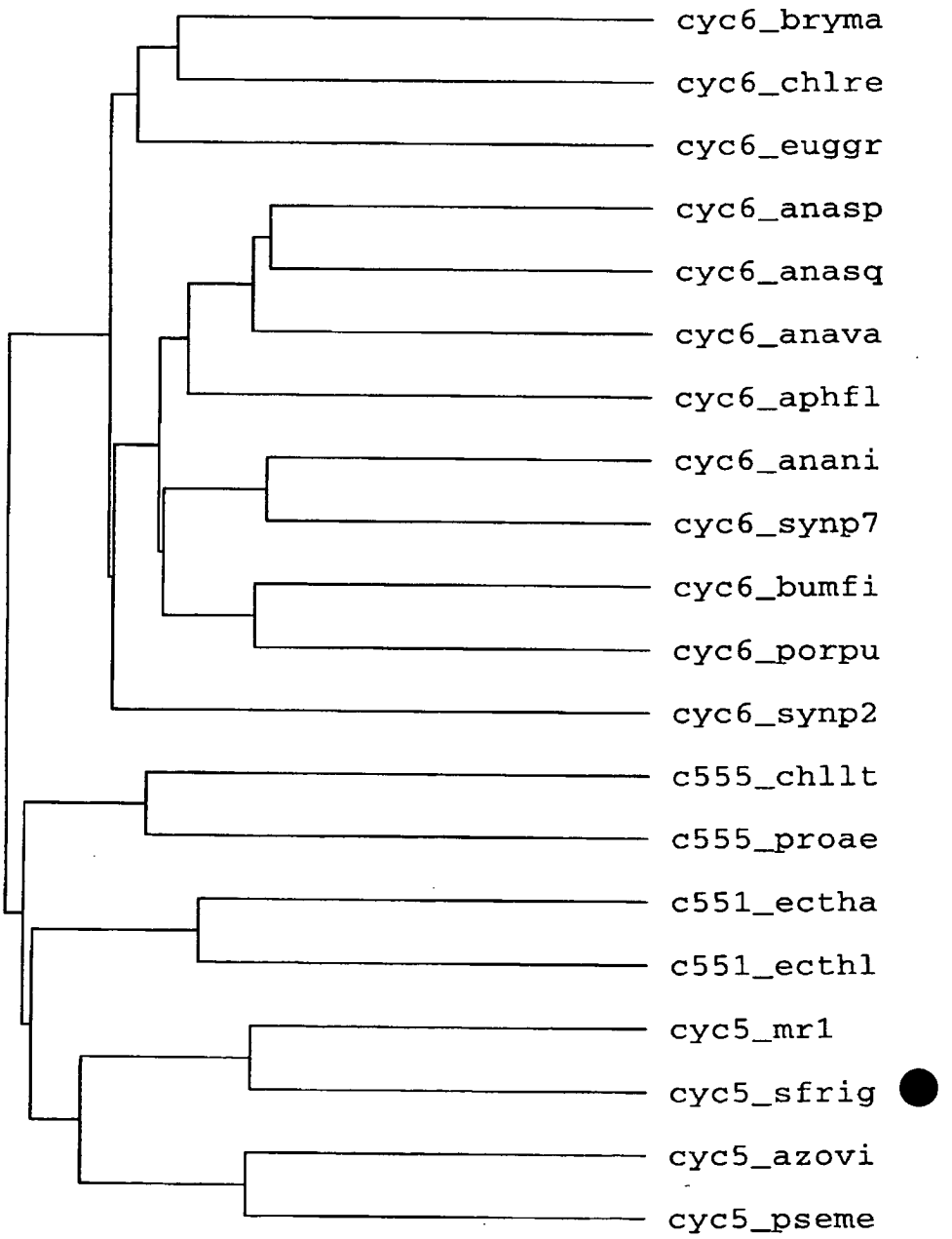
A search for proteins similar to *S. frigidimarina* cytochrome c_5 was made using BLAST (Altschul *et al.*, 1997). The sequence producing the most significant alignment was cytochrome c_5 from *Shewanella putrefaciens* MR-1 which shows 94 % identity (Figure 3.12). *Pseudomonas mendocina* and *Azotobacter vinelandii* cytochromes c_5 show 45 % and 34 % identity, respectively.

Figure 3.12 Results of BLAST using *S. frigidimarina* NCIMB400 *scy*A as the query sequence. Protein with most significant identity given first.

Accession no.	NAME	Protein/ Organism
AF044582	ScyA	Cytochrome c_5/ *Shewanella putrefaciens* MR1
P00121	CYC5_PSEME	Cytochrome c_5/ *Pseudomonas mendocina*
U94420	-	Cytochrome c_5/ *Azotobacter vinelandii*
P11732	CYC5_AZOVI	Cytochrome c_5/ *Azotobacter vinelandii*
P00122	C551_ECTHA	Cytochrome c_{551}/ *Ectothiorhodospira halophila*
P00123	C555_CHLLT	Cytochrome c_{555}/ *Chlorobium limicola*
P38587	C551_ECTHL	Cytochrome c_{551}/ *Ec. halochloris*
P00124	C555_PROAE	Cytochrome c_{555}/ *Prosthecochloris aestuarii*
Q45233	C550_BRAJA	Cytochrome c_{550}/ *Bradyrhizobium japonicum*
P51200	CYC6_PORPU	Cytochrome c_6/ *Porphyra purpurea*
P00103	C551_PSEDE	Cytochrome c_{551}/ *Pseudomonas denitrificans*
P00099	C551_PSEAE	Cytochrome c_{551}/ *Pseudomonas aeruginosa*
P00122	CYC6_MICAE	Cytochrome c_6/ *Microcystis aeruginosa*
P00101	C551_PSEST	Cytochrome c_{551}/ *Pseudomonas stutzeri*
P08197	CYC6_CHLRE	Cytochrome c_6/ *Chlamydomonas reinhardtii*
P00111	CYC6_PORTE	Cytochrome c_6/ *Porphyra tenera*
P24469	CYC6_BACSU	Cytochrome c_{550}/ *Bacillus subtilis*
P00107	CYC6_MONLU	Cytochrome c_6/ *Monochrysis lutheri*

The relationships between these cytochromes may also be depicted as a dendrogram (Figure 3.13). As shown, the closest relations to *Shewanella frigidimarina* cytochrome c_5 are cytochromes c_5 from *Shewanella putrefaciens* MR-1, *Azotobacter vinelandii* and *Pseudomonas mendocina*, followed by the cytochromes c_{551} from *Ectothiorhodospira.* Similarity with cytochromes c_6 from *Porphyra* and *Microcystis* is also shown. Results are in agreement with the classification of the cytochromes c_5, along with cytochromes c_{550}, c_{551} and c_6, in the class I group of cytochromes *c*.

Figure 3.13. Family tree of the class I cytochromes *c*. The dendrogram shows the output of the UWGCG program PILEUP, indicating clusters on the basis of sequence similarity. cyc5_sfrig represents *Shewanella frigidimarina* NCIMB400 *scy*A (marked with red spot); cyc*_name represents "cytochrome c_*" from "species name" as follows: azovi – *Azotobacter vinelandii;* pseme – *Pseudomonas mendocina*; bryma – *Bryopsis maxima*; chlre - *Chlamydomonas reinhardii*; euggr – *Euglena gracilis*; anasp – *Anabaena sp. Strain* PCC7120; anasq – *Anabaena sp. strain* PCC7937; anava – *Anabaena vanaschii*; aphfl – *Aphanizomenon flos-aquae*; anani – *Anabaena nigra*; synp7 – *Synechoccus sp. strain* PCC7942; bumfi – *Bumilleriopsis filiformis*; porpu – *Porphyra purpurea*; synp2 - *Synechoccus sp. strain* p2; chllt – *Chlorobium limicola*; proae – *Prosthecochloris aestuarii* ; ectha – *Ectothiorhodospira halophila* ; ecthl - *Ectothiorhodospira halochloris* ; mrl – *S. putrefaciens* MR-1.



Alignment of the most closely related cytochromes c_5 (Figure 3.14) highlights the conserved features such as the haem attachment motif (C-X-X-C-H) and C- terminal methionine which serves as the sixth haem ligand. The regions surrounding the haem ligands appear to be well conserved throughout the cytochromes c_5, and the overall distribution of charged residues and structurally important residues appears to be very similar (comparisons made with reference to Carter *et al.*, 1985).

The consensus also includes a second pair of cysteine residues which are not involved in haem binding (Figure 3.14).

Figure 3.14 Sequence alignment of various cytochromes c_5. Consensus haem binding domain is highlighted in red, the methionine sixth haem ligand in blue, the signal sequences are highlighted in yellow, and the "extra" cysteines in green (see text). Sequences are labelled as follows: *scy*A_sfrig; *S. frigidimarina*; scyA_sputr, *S. putrefaciens*; cyc5_pseme, *Ps. mendocina*; cyc5_azovi, *Az. vinelandii*. The consensus sequence is also given below the alignment.

```
scyA_sfrig  MKKLLAMTAV AALTMSVNVS AQDAEAIYNK ACTVCHSMGV AGAPKSHYTA
scyA_sputr  MKKLLAMTAV AALTMSVNVS AQDAEAIYNK ACTVCHSMGV AGAPKSHNTA
cyc5_pseme                AASAGGG ARSADDIIAK HCNACHGAGV LGAPKIGDTA
cyc5_azovi                    GGG ARSGDDVVAK YCNACHGTGL LNAPKVGDSA
                                  A        K  C  CH  G    APK    A

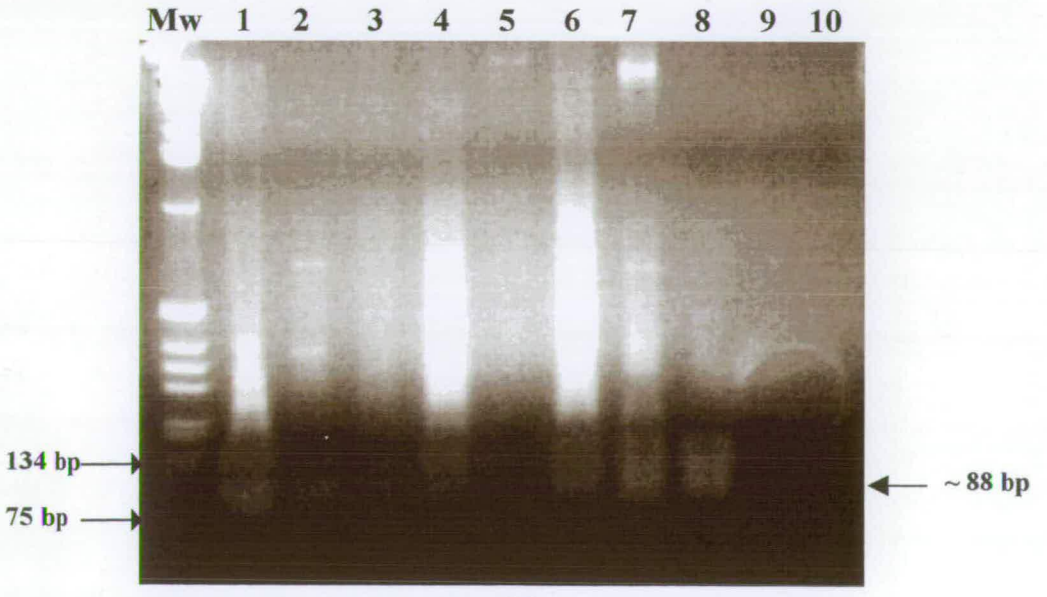
scyA_sfrig DWEPRLAK.. GVDNLVKSVK TGLNAMPPGG MCTDCTDEDY KATIEFMSKAK
scyA_sputr DWEPRLAK.. GVDNLVKSVK TGLNAMPPGG MCTDCTDEDY KAAIEFMSKAK
cyc5_pseme AWKERADHQG GLDGILAKAI SGINAMPPKG TCADCSDDEL REAIQKMSGL
cyc5_azovi AWKTRADAKG GLDGLLAQSL SGLNAMPPKG TCADCSDDEL KAAIGKMSGL
           W  R      G D         GLNAMPP G  C DC D       I  MS
```

3.3.3 Hybridisation studies

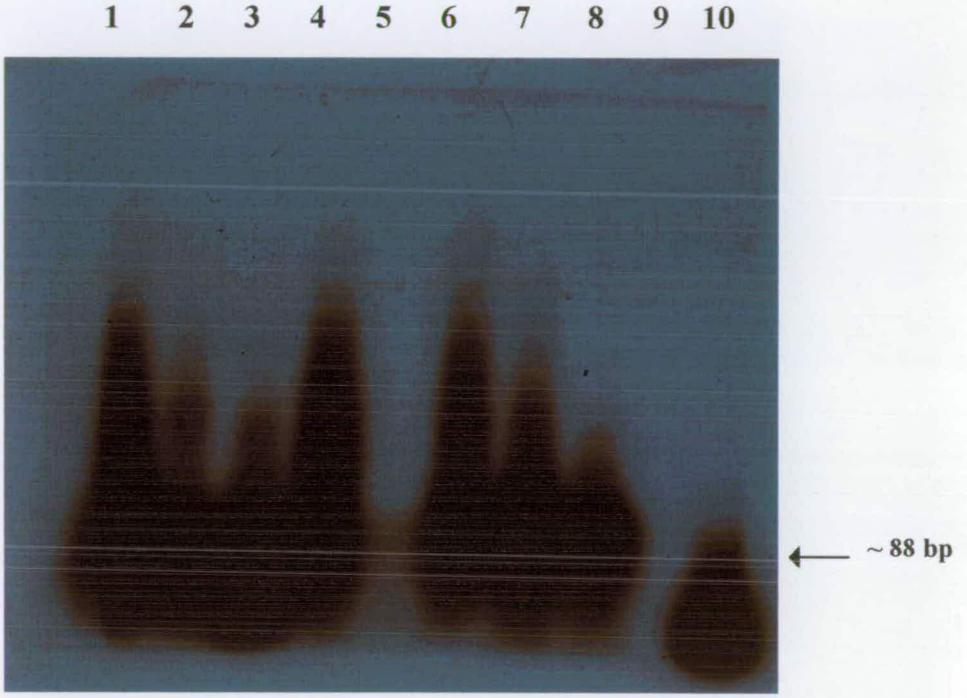
The high degree of sequence similarity between cytochromes c_5 suggests that the protein may be present in other closely related bacterial strains. This hypothesis was tested by polymerase chain reaction and Southern analysis of genomic DNA from *Azotobacter vinelandii, Az. chroococcum, Pseudomonas mendocina, Ps. aeruginosa, Ps. fluorescens, and Ps. putida.* Degenerate oligonucleotides N3187 and N3188 (section 2.1.4) based on the N-terminal region of *S. frigidimarina* cytochrome c_5 were used in PCR reactions. The resulting reaction mixes were separated in 2.0 % agarose gels (Figure 3.15). The expected product size is ~ 88 bp. As shown in figure 3.15A, the putative products could not be separated from "primer-dimer" products. For this reason, a probe, P3129, based on the internal conserved haem binding region was used to probe Southern blots of the PCR products. Results are given in figure 3.15B.

Figure 3. 15 Identification of *scy*A-homologous DNA from different bacteria by PCR analysis and Southern hybridisation. Labels as follows: **1**:*Azotobacter vinelandii,* **2**:*Az. chroococcum,* **3**:*Pseudomonas mendocina,* **4**: *Ps. aeruginosa,* **5**: *Ps. putida,* **6**:*Ps. fluorescens. 7. Shewanella frigidimarina* NCIMB400, **8**. *S. putrefaciens* MR1, **9**. No template DNA, **10**. Primer P3129.

A. Agarose gel electrophoresis of PCR amplified products using primer pair N3187 and N3188; Reaction cycles as follows: 1 x 94°C, 3 mins.; 35 x 94°C, 1min; 35 °C, 1 min; 72°C, 1 min., and 1 x 72°C 10 mins.. Reactions were performed in a solution containing 100 ng genomic DNA, 10 pmol each primer, 200 µM dNTPs, and 1 Unit *Tbr* DNA polymerase in 1 x reaction buffer. Products were separated in a 2.0 % agarose gel.



B. Autoradiograph of a Southern blot of PCR products probed with γ^{32}P-labelled P3129. The blot was washed twice in 2x SSC; 0.1 % SDS at room temperature for 15 mins before exposure. PCR products following reactions with no template DNA, and primer P3129 were used as negative and positive controls (lanes 9 and 10 respectively).



Strong signals were observed for *Azotobacter chroococcum, Az. vinelandii, Pseudomonas aeruginosa, Ps. fluorescens, Ps. Mendocina* and both *Shewanella* strains. No hybridising bands were observed for *Ps. putida* which suggests that cytochrome c_5 is either not present or the coding sequence is not sufficiently similar for hybridisation in this strain. No PCR product or hybridisation was seen in the control lane in which contained PCR reaction mix with no DNA template.

Southern blots of genomic DNA from all of the strains above were also probed using P3129. No hybridising bands were detected for *Ps. putida*, or for *Ps. mendocina*. The PCR step was needed to detect the cytochrome c_5 homologue in *Ps. mendocina*.

Such PCR and hybridisation techniques have been used previously to assess the relatedness of cytochrome c_3 genes from a variety of *Desulfovibrio* strains (Kwoh *et al.*, 1993) and also to detect *fix*NOPQ genes in a variety of bacteria (Thöny-Meyer *et al.*,

1994). The success of the technique, as demonstrated here, establishes the utility of PCR for quickly and easily identifying organisms that contain closely related genes.

3.4 Northern blot analysis of *scy*A

In the absence of any obvious regulatory regions proximal to the coding sequence for the cytochrome c_5 structural gene, and the lack of any putative co-transcribed proteins, Northern blot analysis was used to examine expression and the size of the RNA message encoding ScyA.

To this end, total RNA was extracted from both aerobically and anaerobically grown *Shewanella frigidimarina* NCIMB400, denatured, separated by electrophoresis, and blotted onto nitrocellulose. An internal gene fragment of *scy*A was generated by PCR, labelled by nick translation, and used as a probe (Figure 3.16).

Figure 3.16 Northern blot analysis of *scy*A expression. **A**. Probe design. Sequences for primers Mc5f and Mc5r are given in section 2.1.4. **B**. Agarose gel analysis of PCR products using Mc5f and Mc5r; PCR cycles were performed in a single chemical synthesis as follows: Reaction cycles as follows: 1 x 94°C, 3 mins.; 35 x 94°C, 35 s; 50°C, 45 s; 72°C, 1 min., and 1 x 72°C 10 mins.. Reactions were performed in a solution containing 100 ng genomic DNA, 11 pmol each primer, 200 μM dNTPs, and 1 Unit *Taq* DNA polymerase in 1 x reaction buffer. Products were separated in a 1.2 % agarose gel. Gibco-Brl 1 kb ladder used as size standards.

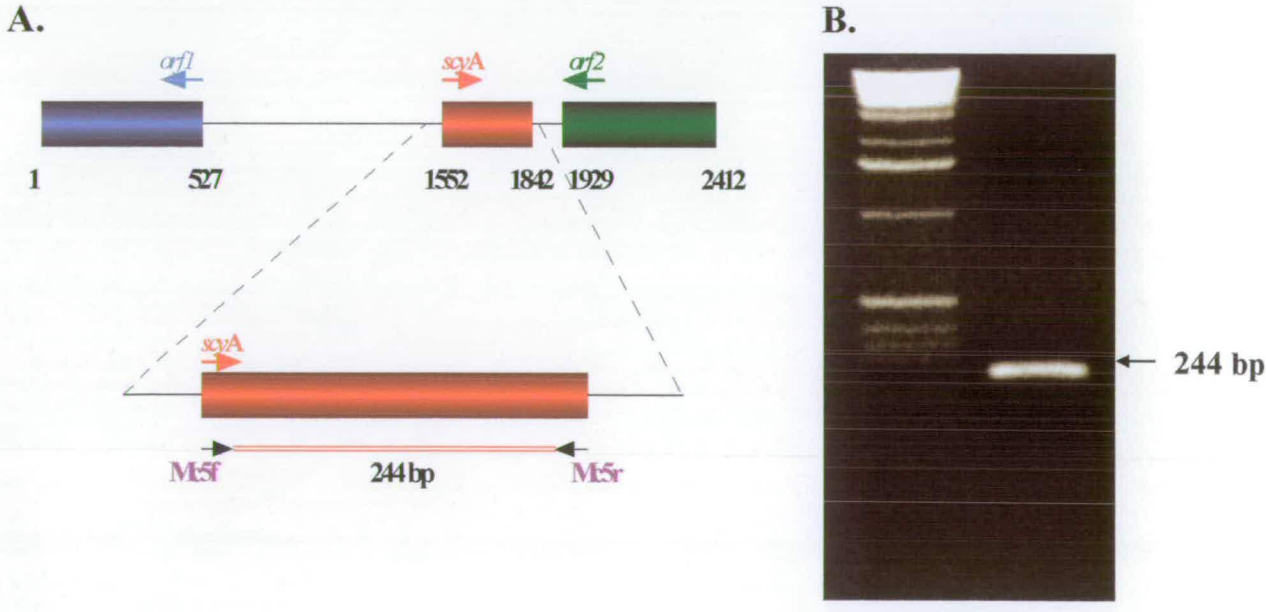
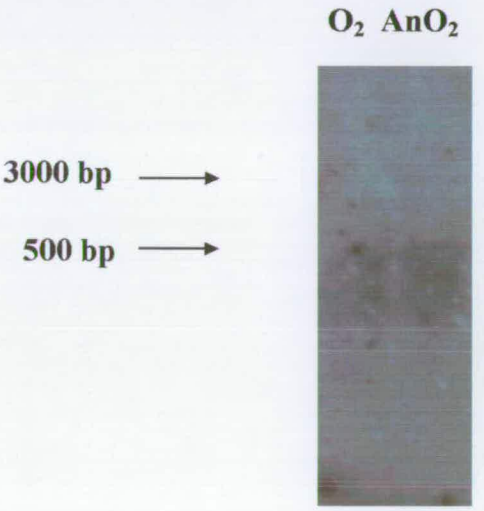


Figure 3.17 shows the autoradiograph of probed RNA. The size of the transcripts was determined relative to RNA molecular weight markers. The DNA probe hybridised with

a transcript of ~600 nt in equal amounts with RNA from both aerobically and anaerobically grown cultures.

Figure 3.17 Autoradiograph of a Northern blot of total RNA extracted from aerobically or anaerobically cultured *Shewanella frigidimarina* NCIMB400. Blots were hybridised at 42 °C for 8 hours and washed successively in 2 x SSC; 0.1% SDS, 0.5 x SSC; 0.1% SDS, and 0.1 x SSC; 0.1%SDS, at room temperature for 15 mins. before exposure. M_w was determined by ethidium bromide staining of NEB RNA markers.



The nucleotide sequence coding for the cytochrome c_5 structural gene has been determined as 280 bp (Section 3.2). As shown here, a probe based on the internal sequence of *scy*A hybridises to an approximately 500-600 nt RNA strand. These results provide further evidence that *scy*A is not cotranscribed with any other genes, and also agree with the finding that cytochrome c_5 may be extracted from both aerobic- and anaerobically grown *S. frigidimarina* (Morris, 1987).

3.5 Overexpression of cytochrome c_5

A recurring feature of the cytochromes c_5 is the presence of an additional pair of cysteine residues toward the C-terminal end of the amino acid sequence (Figure 3.13). These cysteines are not involved in haem-binding and the reason for their conservation remains unknown. Such an additional pair of cysteines has been identified in calf thymus glutaredoxin where they are thought to possess some regulatory significance (Klintrot *et al.*, 1984).

In order to investigate the role, if any, of the additional pair of cysteines in *S. frigidimarina* cytochrome c_5, a suitable expression system must first be generated. Once established, site directed mutagenesis may be performed to replace either, or both, of the cysteines and enough recombinant protein generated for biochemical characterisation.

3.5.1 Site Directed Mutagenesis

To facilitate cloning of the cytochrome c_5 structural gene into expression vectors, a number of restriction sites had to be introduced into the insert containing the gene. The restriction sites are designed to allow cloning into the multiple cloning site of a number of vectors in such a way as to optimise the distance between the vector-encoded ribosome binding site and the start codon of the insert-encoded gene.

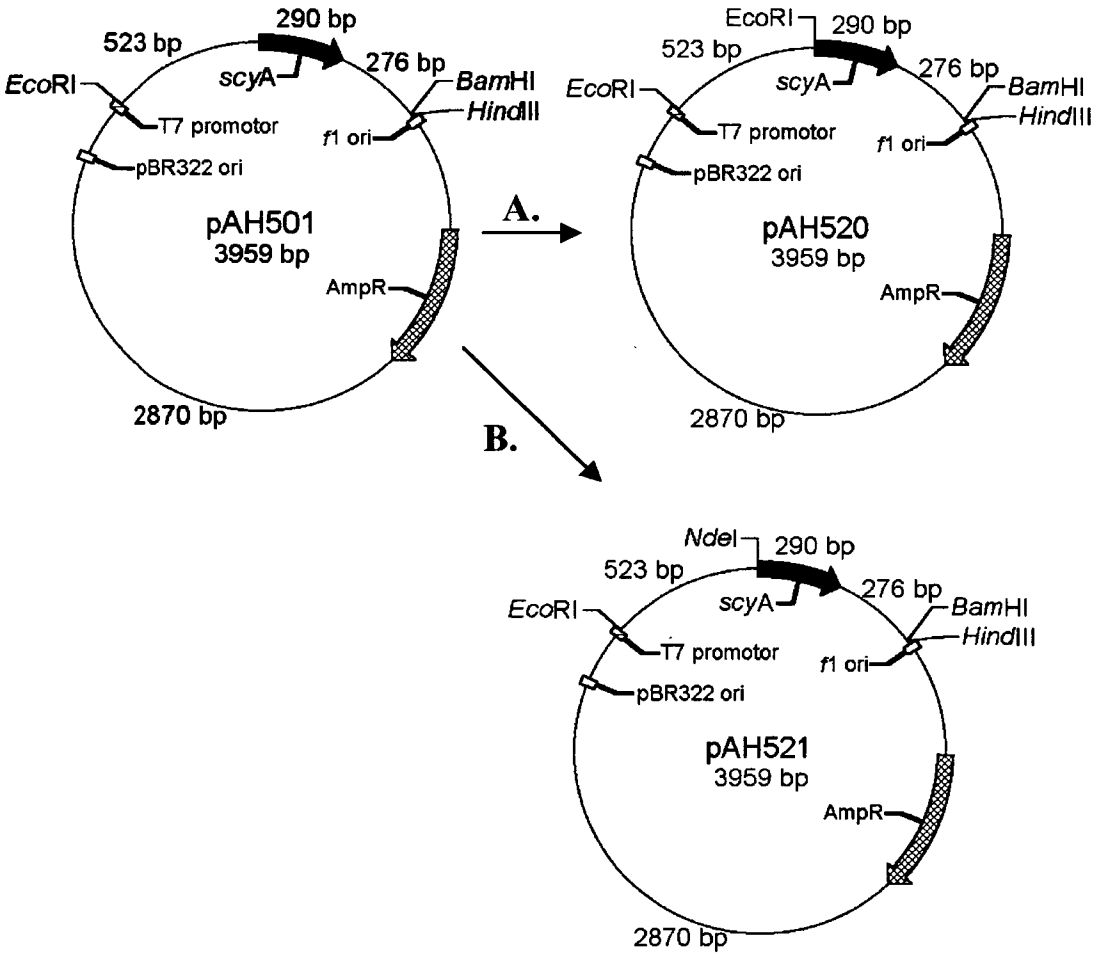
To this end, single stranded template DNA was generated from plasmid pAH501, which consists of a 1089 bp region of PCR amplified DNA encoding the cytochrome c_5 structural gene cloned directionally into the phagemid vector pTZ18R (Figure 3.5). Plasmid pAH501 was first transformed into *E. coli* strain BW313, a *dut*, *ung* host (for complete genotype see 2.1.1). Following infection of the transformed cells with helper phage and overnight growth, single stranded DNA was extracted. As a result of growth in BW313 the single stranded template DNA is uracil-enriched. This aids mutagenesis

according to the method of Kunkel *et al.* (1985), the method used for the incorporation of the restriction sites in this study.

An *Eco*RI site was first introduced into pAH501, as shown in figure 3.18A, using oligonucleotide C5EcoRI (2.1.4), to create plasmid pAH520.

Similarly, plasmid pAH521 was created by introducing an *Nde*I site using primer C5NdeI (Figure 3.18B).

Figure 3.18. Introduction of restriction sites into pAH501 using site directed mutagenesis. **A**. *Eco*RI site, to create pAH520. **B**. *NdeI* site, to create pAH521. SDM was performed as described (chapter 2), annealing at 70 °C for 5 minutes.



Downstream of the *scy*A stop codon, a primer C5STOP was designed to introduce *Bam*HI and *Hind*III sites into pAH501. A range of temperatures, varying from 65 °C to 95 °C was used for annealing reactions; however no mutations were introduced. The inability to create mutations in this region provides further evidence for the likelihood of formation of the proposed termination structure in the *scy*A transcript (Figure 3.19).

Figure 3.19 Details of the region on which primer C5STOP was designed. The region which the primer covers is highlighted in blue. The TAA stop codon for *scy*A is highlighted in red.

Plasmids pAH520 and pAH521 contain *Bam*HI and *Hind*III sites, from the multiple cloning site of pTZ18R, 276 bp downstream of the termination structure which may be used for cloning purposes (Figure 3.18).

Before further use of either pAH520 or pAH521, the region encoding *scy*A in both plasmids was sequenced to ensure no secondary mutations had been introduced.

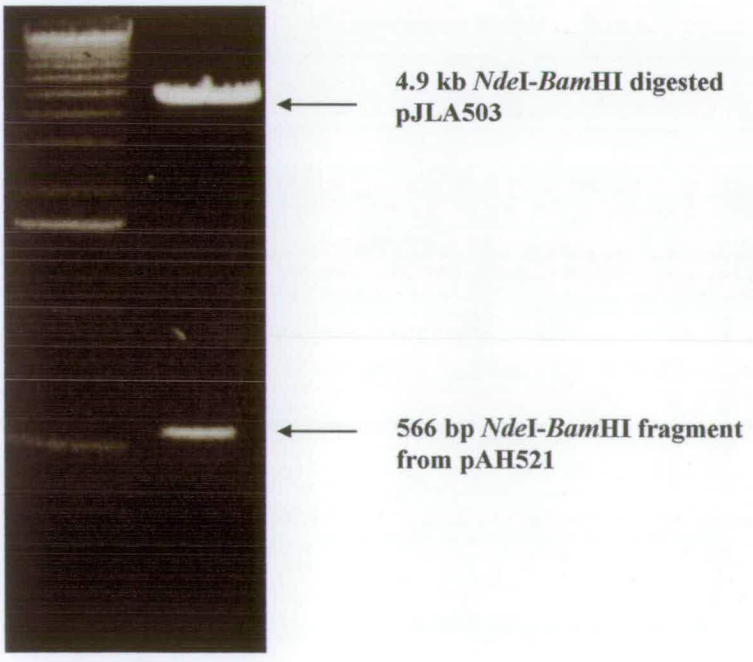
3.5.2 Expression of *S. frigidimarina* cytochrome c_5 in *Escherichia coli*

3.5.2.1 pJLA503

The expression vector pJLA503 contains the thermo-inducible λ P_L and P_R promoters, which are repressed by the λ c^{I857} protein supplied by *E. coli* strains such as NF1 (2.1.1; Stanley and Luzio, 1983). The c^{I857} protein represses the P_L and P_R promoters at a temperature of 30 °C and lower, but when the temperature is shifted to 42 °C, the repressor protein is inactivated and the promoter is induced.
Plasmid pJLA503 also contains a unique cloning site for *Nde*I for expression of non-fused genes and unique promoter distal cloning sites *Bam*HI, *Eco*RI, *Sal*I and *Sph*I, along with an ampicillin resistance cassette for selection (Chapter 2).

To express ScyA, the coding region was excised from pAH521 on an *Nde*I-*Bam*HI fragment and cloned directionally into pJLA503, which had been similarly digested, to create plasmid pAH530 (Figure 3.20)

Figure 3.20. Agarose gel electrophoresis of plasmid pAH530 following 3 hours digestion with *Nde*I and *Bam*HI at 37 °C.



Once constructed, plasmid pAH530 was transformed into *E. coli* strains B834, BL21(DE3), BDS, BL23, DH5α, JM109(DE3), K12, NF1, TG1, and POP2136 (for genotypes see 2.1.1). Of these strains only NF1 and POP2136 are "cIts", the remaining strains were used to examine basal expression with pJLA503 as the expression vector and also to serve as controls for temperature induction experiments. Transformants were verified by isolation of plasmid DNA, and growth of strains containing the plasmid on media supplemented with ampicillin.

Each strain harbouring pAH530 was cultured in Luria Bertani medium, supplemented with 100 μg/ ml ampicillin, at 30 °C until the $O.D_{600}$ reached ~ 0.6. The temperature was then shifted to 42 °C. Following either 6 hours or overnight induction, cells were harvested and soluble fractions analysed by SDS PAGE and Coomassie blue staining.

Ubbink *et al.* (1992) reported that for expression of *Thiobacillus* cytochrome c_{550} in *E. coli*, semi-anaerobic growth conditions resulted in elevated expression levels in comparison with aerobic or anaerobic cultures. For this reason, all strains harbouring expression vectors for cytochrome c_5 were grown under semi-anaerobic conditions.

Examination of both Coomassie blue and haem-stained gels indicated that there is little or no overexpression of cytochrome c_5 using pJLA503 as the expression vector (results not shown). Indeed no expression is expected using the strains B834, BL21(DE3), BDS, BL23, DH5α, JM109(DE3), K12, and TG1 with this plasmid.

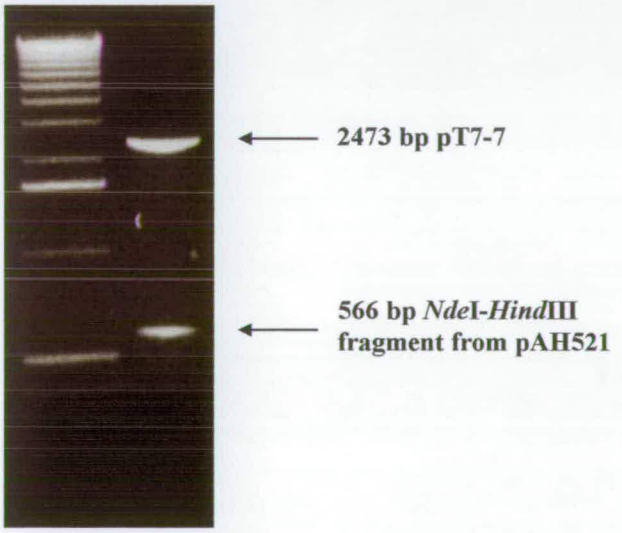
The relatively low cost of temperature induction in comparison with chemically induced expression vectors has led to an increase in the use of vectors containing temperature dependent promoters, particularly in industry.

3.5.2.2 pT7-7

The expression vector pT7-7contains a strong promoter from bacteriophage T_7. Vectors containing the T7 promoter rely on the T7 RNA polymerase gene, usually supplied by the host (λDE3 lysogen), for expression of target proteins. In λDE3 lysogens, the T7 RNA polymerase is under the control of the *lac*UV5 promoter. This allows some degree of transcription even in the uninduced state, and in the absence of further controls is suitable for expression of many genes whose products have innocuous effects on host cell growth.

The cytochrome c_5 structural gene was cloned into pT7-7 on an *Nde*I-*Hind*III fragment from pAH521, to create the recombinant plasmid pAH531 (Figure 3.21).

Figure 3.21. Plasmid pAH531 digested with *Nde*I and *Hind*III.



Recombinant plasmid pAH531 was then transformed into *E. coli* strains B834, BL21(DE3), BDS, BL23, BW313, DH5α, JM109(DE3), K12, NF1, TG1, and POP2136 (for genotypes see 2.1.1). Transformants were again verified by extraction of the correct plasmid DNA.

As with pAH530, expression was examined by Coomassie Blue- and haem-staining of SDS-PAGE gels (results not shown). As with expression by pJLA503, no prominent bands were noted with pT7-7 as the expression vector.

The T7 promoter of pT7-7 is known to be not regulated. For this reason *scy*A was cloned into the pET-vectors pET11a and pET19, which have tightly regulated promoters.

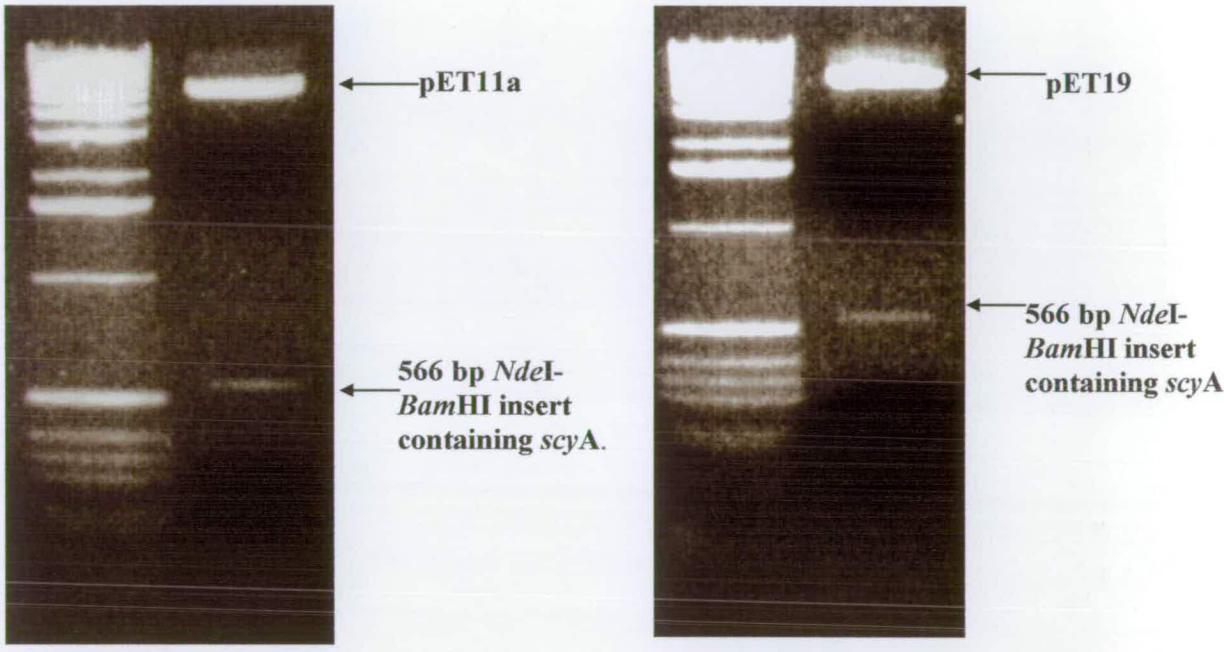
3.5.2.3 The pET system

The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli.* The vectors are derived from pBR322, based on the T7 promoter-driven system described above, and include over 30 vector types. Instead of the "plain" T7 promoter found in pT7-7, the pET vector series contain the T7*lac* promoter. The T7*lac* promoter contains a 25 bp *lac* operator sequence downstream of the promoter region. Binding of the *lac* repressor, supplied by the plasmid, at the *lacO* site effectively reduces transcription by T7 RNA polymerase, thus providing a second *lacI* based mechanism to suppress basal expression in λDE3 lysogens.

In this study, two pET vectors were used. The first was pET11a, which contains an 11 amino acid T7-gene10 NH_2-terminal tag sequence. The N-terminal tag may be used for immunoprecipitation, immunofluorescence or Western blotting. The second was pET19, which contains an N-terminal HIS-Tag sequence which may be useful in various purification procedures.

To clone *scy*A into both pET11a and pET19, the structural gene was excised from pAH521 on an *Nde*I-*Bam*HI fragment and cloned directionally to create recombinant plasmids pAH532 and pAH533, respectively (Figure 3.22).

Figure 3.22. Agarose gel electrophoresis of plasmids pAH532 and pAH533 digested with *Nde*I and *Bam*HI. 0.8% agarose. Gibco-BRL 1 kb markers are used as size standards.



Plasmids pAH532 and pAH533 were transformed into *E. coli* strains B834, BL21(DE3), BDS, BL23, DH5α, JM109(DE3), K12, NF1, TG1, and POP2136 (for genotypes see 2.1.1). Following verification, these strains were grown microaerobically at 37 °C until the Absorbance at 600 nm reached ~ 0.6. Cultures were induced with 0.3 mM IPTG for 6 or 18 hours, harvested and the soluble fractions analysed by SDS-PAGE followed by Coomassie- and haem-staining. No bands were observed at the correct position.

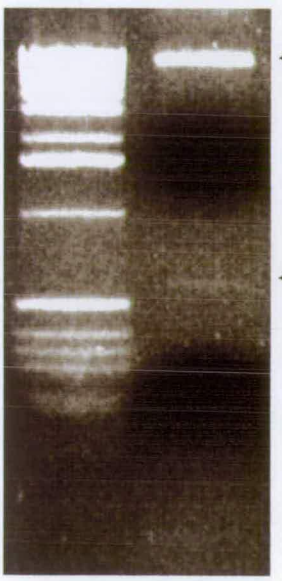
3.5.3 Overexpression of cytochrome c_5 in *Shewanella frigidimarina* NCIMB400

Frequently researchers experience low protein yields in *E. coli* even when a strong promoter, such as T7, are used. A second problem encountered when expressing proteins in a foreign host is incorrect processing of the protein of interest; the protein may not be folded properly, or appropriate prosthetic groups added incorrectly. Expression in *E. coli* is, however, often the only method of producing enough protein for biochemical characterisation due to the knowledge we have of *E. coli* genetics. An alternative is to construct an expression vector which may be introduced into the native host.

Recently, a broad-host range vector was constructed for Gram negative bacteria (Overbye *et al*, 1995). This vector, pMMB503, contains a mob system and a single replicon, IncQ, which exhibits a particularly wide host range that includes the majority of Gram negative bacteria, and more importantly a number of *Shewanella* strains. In this study, pMMB503 was used to overexpress cytochrome c_5 in *Shewanella frigidimarina* NCIMB400.

Initially, the *scy*A gene was excised from pAH520 on an *Eco*RI-*Hind*III fragment and cloned directionally into pMMB503 which had been similarly digested, to create plasmid pAH534 (Figure 3.23). This recombinant plasmid was then transformed into ultracompetent *E. coli* strain SM10.

Figure 3.23 Agarose gel electrophoresis of plasmid pAH534 digested with *Eco*RI and *Hind*III (3 hours, 37 °C)



Following construction, plasmid pAH534 had to be introduced into *S. frigidimarina* NCIMB400. This was achieved by conjugal mating of an *Hfr* (*h*igh *f*requency of *r*ecombination) strain of *E. coli*, SM10, harbouring pAH534, with wild type *S. frigidimarina* NCIMB400. Both *E. coli* donor and *S. frigidimarina* recipient cells were cultured to exponential phase then mixed at a ratio of approximately 10^6 donor cells and 10^7 recipient cells. The mixture was plated on Luria Bertani medium supplemented with rifampicin and streptomycin to select for *S. frigidimarina* containing the expression vector (plasmid pMMB503 contains a streptomycin resistance cassette; *S. frigidimarina* is rifampicin resistant). Typically a mating reaction yielded over 100 exconjugant cells after incubation for 3 days at 23 °C. No spontaneous mutants were ever isolated from control plates containing solely *S. frigidimarina* wild-type cells, or *E. coli* SM10 harbouring pAH534, plated on the same medium. This implies that exconjugant cells had acquired pAH534.

Four colonies, selected at random and named strain AH500, were used for expression analysis. As with the above expression vector clones, overexpression was examined through SDS-PAGE electrophoresis followed by Coomassie- and haem-staining (Figure 3.24).

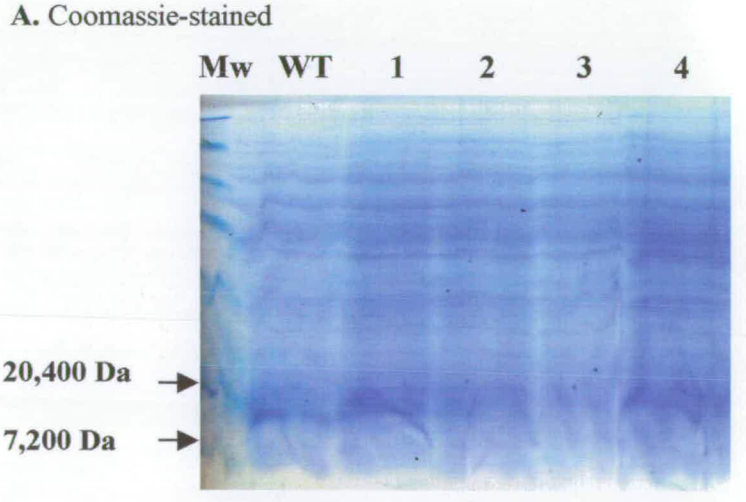
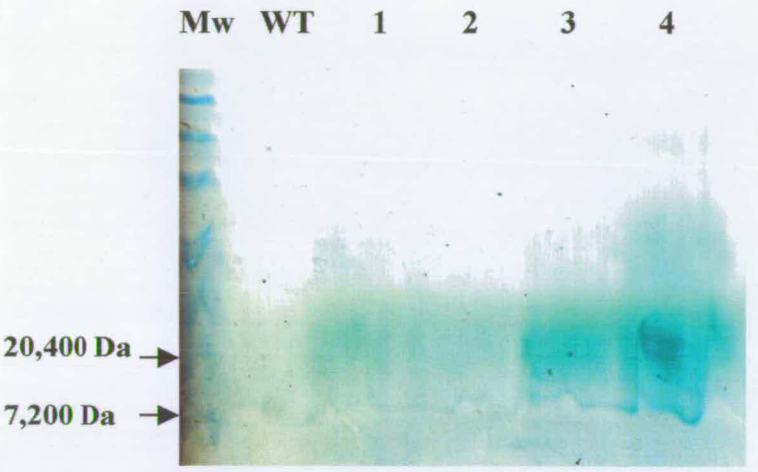


Figure 3.24 12% SDS-PAGE of soluble fractions of Wild-type *S. frigidimarina*, and strain AH500. Mw - Biorad prestained broad range protein markers; wt - wild-type; Lanes 1-4 contain soluble fractions of four randomly chosen colonies of strain AH500. Fractions in lanes 1-2 were isolated from uninduced cultures. Those in lanes 3-4 were induced overnight with 0.3 mM IPTG.

Figure 3.24 B. Haem-stain



In Coomassie stained gels (Figure 3.24A), differences in expression levels of cytochrome c_5 are difficult to estimate. However, using haem staining (Figure 3.24B) an obvious rise in expression of a cytochrome *c* of the molecular weight of cytochrome c_5 is represented by a deeper-coloured band in recombinant strains with respect to wild-type.

In the recombinant plasmid, pAH534, the cytochrome c_5 gene comes under the control of the *tac* promoter. The inclusion of IPTG in cultures led to an increase in expression (lanes 3-4, Figure 3.24B).

Expression of cytochrome c_5 in strain AH500 was also examined during large scale preparation of the protein. Levels of protein obtained using this expression system rose from approximately 0.04 mg / litre of culture in wild-type *S. frigidimarina* NCIMB400 to approximately 0.75 mg / litre of culture in strain AH500; an increase of over 15-fold (Doherty and Clark, unpublished results). N-terminal sequencing of the recombinant protein has also confirmed that it is cytochrome c_5.

3.6 Discussion

The class Ib cytochromes *c* are characterised by their small size, and the possession of a single haem group toward the N-terminus. In line with this classification, the inferred amino acid sequence of the *Shewanella frigidimarina* NCIMB400 cytochrome c_5 corresponds to a mature protein sequence of 78 amino acids with a single haem attachment peptide located at residues 11 to 15 and a methionine at position 53 which serves as the sixth haem ligand. The calculated molecular weight of the holoprotein is 8910.5 Da.

Early studies on both of the cytochromes c_5 from *Pseudomonas mendocina* and *Azotobacter vinelandii* showed that these proteins displayed N-terminal heterogeneity in a sequence rich in alanine and glycine residues (Ambler, 1974; Meyer and Kamen, 1982). This led to the worry that cytochromes c_5 are derived by proteolysis of a larger protein, such as cytochrome *c* peroxidase or cytochrome *cd*. Results described here, however, show that *Shewanella frigidimarina* cytochrome c_5 is transcribed on a single mRNA strand as a 99 amino acid polypeptide with a typical N-terminal prokaryotic signal sequence. Similarly, cytochromes c_5 from *S. putrefaciens* MR-1, and also *Az. vinelandii,* have been shown to be transcribed as individual polypeptides, rather than as part of a larger protein (Saffarini *et. al*, 1998; Rey and Maier, 1997).

Wood (1983) proposed that all *c*-type cytochromes are located either in the periplasm or, in Gram negative organisms, on the periplasmic face of the inner membrane. This hypothesis has been central to the understanding of how *c*-type cytochromes are synthesised. Over the last few years a wealth of evidence has been generated to show that *c*-type cytochromes are formed as apoproteins in the cytoplasm which mature during or after a membrane translocation step in which the enzyme haem-lyase inserts the haem covalently. In this chapter several pieces of evidence support the periplasmic location of cytochrome c_5. First, it was observed that the *scy*A open reading frame contains a 21 amino-acid N-terminal sequence that is missing in purified cytochrome c_5. This N-

terminal extension conforms to the format of a typical periplasmic signal sequence, suggesting that cytochrome c_5, like other *S. frigidimarina* soluble cytochromes, is made as a higher molecular weight precursor that is exported from the cytoplasm (Pealing, 1994; Gordon, unpublished). Haem staining for cytochrome c_5 also shows the protein to be localised to the soluble fraction and hydropathy plotting shows the protein to be predominantly hydrophilic. These results do not agree with those of Hunter *et al.* (1989), who provide evidence that cytochromes c_5 from *Azotobacter vinelandii* and *Pseudomonas stutzeri* are predominantly membrane bound.

Members of the cytochrome c_5 group are unique in having an extra pair of cysteine residues, toward the C-terminal end of the protein, which are not used in haem binding. These residues are strictly conserved, and their function is still unknown. Within the bacterial glutaredoxins, an extra cysteine pair may also be found. Here, they are suspected to possess some regulatory significance (Klintrot *et. al*, 1984). In *Azotobacter*, the extra pair of cysteines forms a closed local disulphide loop (Carter *et al.*, 1985). The function of the disulphide may be to stabilise the structure.

The region upstream of the coding sequence for *S. frigidimarina* cytochrome c_5 was examined for possible regulatory sites. A putative EtrA box was found 273 bp upstream of the ATG start site. This site clearly shows a high degree of similarity to the consensus FNR box found in *E. coli* and a variety of other organisms (Table 3.1). In *E. coli* however, the FNR sites are usually positioned around 40 bp upstream of the transcriptional start site, for genes positively regulated by FNR; for negatively regulated genes the FNR box usually overlaps the transcript start site. Abnormal spacing of FNR boxes has been reported in *Pseudomonas* G-179 where the FNR site for regulation of the *nir*U gene is 300 bp upstream of the transcript start site (Ye *et al.*, 1993). Indeed, a putative EtrA site has been found for *S. frigidimarina* flavocytochrome c_3 at a position of -153 bp (Gordon, 1996).

Regulation of *Azotobacter vinelandii* cytochrome c_5 (*cycB*), has been examined using *cycB-LacZ* fusions (Rey and Maier, 1997). Levels of β-galactosidase activity were tested

upon culturing cells in a range of oxygen tensions from 1.5% partial pressure to fully aerobic. Under all conditions little difference in activity was noted. In line with these results, Northern blot analysis of *scy*A from *S. frigidimarina* has shown that expression levels are identical in both aerobically and anaerobically grown cultures.
Further examination of *cyc*B regulation by Rey and Maier (1997) has focused on the effect of nitrogen. Expression of *cyc*B has been shown to be highly correlated with nitrogen fixation. As suggested by the authors, the up-regulation upon nitrogen starvation could be due to regulatory elements such as *nif*A, or alternatively a factor such as the *Klebsiella* nitrogen assimilation control (NAC) protein that couples transcription of σ^{70}-dependent promotors to regulation by the nitrogen regulatory system. Expression of *S. frigidimarina* cytochrome c_5 is discussed further in chapter 4.

It is only very recently that nucleotide sequence information has become available for the cytochromes c_5 enabling a more detailed examination of their role(s) and information concerning their evolution. Comparison of sequences available for *Shewanella frigidimarina* NCIMB400 and *S. putrefaciens* MR-1 cytochrome c_5 show an extremely high degree of similarity (94 %). This implies a relatively recent divergence of these strains. Indeed, their classification as distinct species has been a matter of debate for a number of years. Relatedness of *S. frigidimarina* cytochrome c_5 to other cytochromes c_5 may be demonstrated very readily through the use of PCR and hybridisation. As shown here, by designing PCR probes and primers corresponding to both conserved and non-conserved regions of a protein, it is possible to obtain an estimate of relatedness between strains. In this case, the cysteine residues, on which primer P3129 is based, are highly conserved and therefore represent the areas of highest probability for sequence similarity in the protein.

Overproduction of several cytochromes *c* has been attempted in *E. coli*, and a number of problems have been encountered. These range from the formation of inclusion bodies to insufficient amounts for purification, and also lack of incorporation of haem (Grisshammer *et al.*, 1994; Self *et al.*, 1990, Wachenfeldt and Hederstedt, 1990). Expression of *S. frigidimarina* cytochrome c_5 in *E. coli* was attempted using the

expression vectors pJLA503, pT7-7, pET11a, and pET19. Examination of SDS-PAGE gels following both Coomassie blue or haem staining indicates that cytochrome c_5 is not effectively overexpressed using any of these expression vectors. Reasons for lack of expression in the pET series of expression vectors is unknown. A number of factors may be responsible such as induction conditions, incorrect alignment of start codon, or mRNA stability. Fine-tuning of the translational initiation region of the signal sequence may have an effect on expression (Simmons and Yansura, 1996).

Overproduction of cytochrome c_5 within *S. frigidimarina* was also attempted using the *tac* expression vector pMMB503. High level expression of cytochrome c_5 was achieved using this system.

The development in this study of cytochrome c_5 expression systems sets the stage for production of recombinant protein following alteration of the extra pair of cysteines.

Chapter 4

Cytochrome *c*$_5$ from *Shewanella frigidimarina* NCIMB400: Gene Disruption and Phenotypic Characterisation

The first characterised cytochrome c_5 was isolated from *Azotobacter vinelandii* in 1956 (Tissières and Burris, 1956, Tissières, 1956). Since then a number of cytochromes sharing significant amino acid similarity, high redox potential, and low molecular weight have been isolated from a variety of *Pseudomonas sp.* and, more recently from *Shewanella sp.* (Ambler and Taylor, 1973; Saffarini *et al.*, 1998). Although extensive biochemical and biophysical characterisation of these cytochromes has been carried out, genetic information is only now becoming available. It is this genetic information which enables further examination of the physiological role(s) of these cytochromes.

In bacterial genomes there is a tendency for proteins of related function to be co-transcribed in multicistronic operons. Examination of the region encoding *Shewanella frigidimarina* cytochrome c_5 has, however, shown that *scy*A, the structural gene, is not co-transcribed with any other protein coding sequences (Chapter 3). An alternative means to elucidate the role of a particular protein is to inactivate the endogenous gene. Comparison of the phenotype of the resulting deletion strain with a wild type strain subsequently can enable identification of the physiological role of the protein.

Work in this chapter describes the construction of a strain of *Shewanella frigidimarina* NCIMB400 in which the structural gene encoding cytochrome c_5 has been deleted from the chromosome. Results of phenotypic characterisation of the deletion strain are then presented.

4.1 Targeted disruption of cytochrome c_5 in *Shewanella frigidimarina* NCIMB400

The principle of gene disruption is to use homologous recombination to replace the endogenous chromosomal copy of a gene with an inactivated copy. To this end, the *S. frigidimarina scy*A gene was disrupted by exchanging a 205 base pair *Psh*AI-*Sex*AI restriction endonuclease fragment with an approximately 2000 bp streptomycin/ spectinomycin resistance cassette (ΩSm; Figure 4.1).

Plasmid pAH501, which contains *scy*A on a 1089 bp PCR fragment cloned into pTZ18r, was used as the template for insertion of ΩSm. The *scy*A gene contains unique sites for *Psh*AI and *Sex*AI at positions 15 and 220, respectively (numbering relative to ATG start site for *scy*A). Digestion with these endonucleases removes a 205 bp region of DNA. This represents deletion of 71 % of the coding sequence.

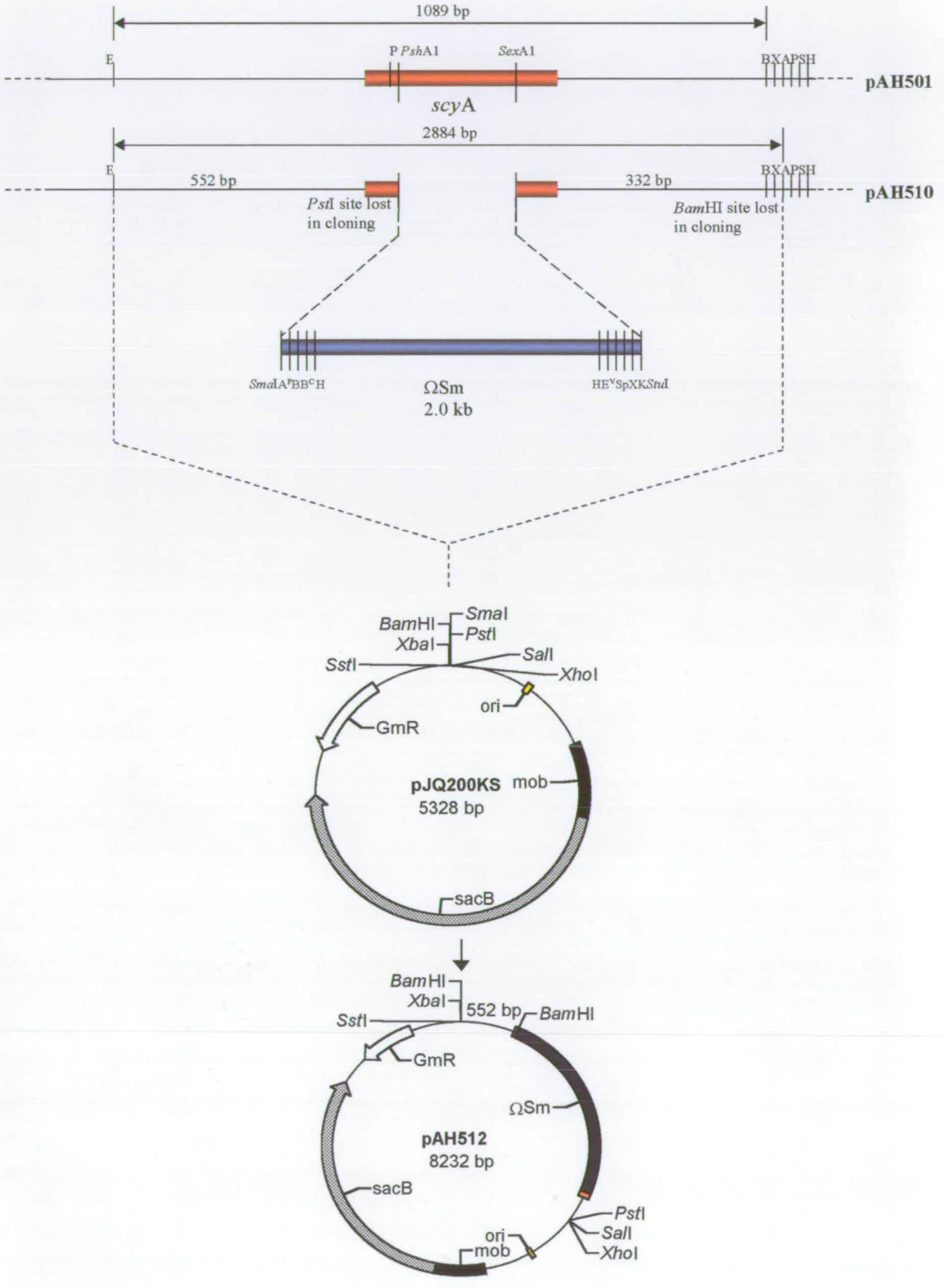
Before digestion, pAH501 had to be transformed into *E. coli* strain BL21(DE3), and plasmid DNA extracted from this host. The restriction endonuclease *Sex*AI is *dcm* methylation sensitive, and will only digest unmethylated DNA such as that extracted from a *dcm* host like BL21(DE3) (for full genotype see section 2.1.1).

Digestion with *Sex*AI and *Psh*AI was carried out sequentially, precipitating the DNA with ethanol between each digestion. Neither restriction enzyme cuts efficiently, and reaction mixes required overnight incubation at 37 °C.

Following digestion, the 5' cohesive terminus generated by *Sex*AI was blunt-ended using T4 DNA polymerase. Digestion with *Psh*AI creates a blunt end. Into this site a streptomycin/ spectinomycin resistance cassette was ligated (Figure 4.1). The 2000 bp resistance cassette was obtained from pHRP317 on a *Sma*I-*Stu*I fragment (Parales and Harwood, 1993; Prentki and Krisch, 1984). The recombinant plasmid containing the *scy*A::ΩSm construct in pTZ18r was designated pAH510. Transformants containing pAH510 were selected for by plating on LB supplemented with streptomycin and ampicillin.

In order to introduce the deletion construct into *Shewanella frigidimarina* it had to be transferred from pTZ18r to the suicide vector pJQ200KS (Figure 4.1). This suicide

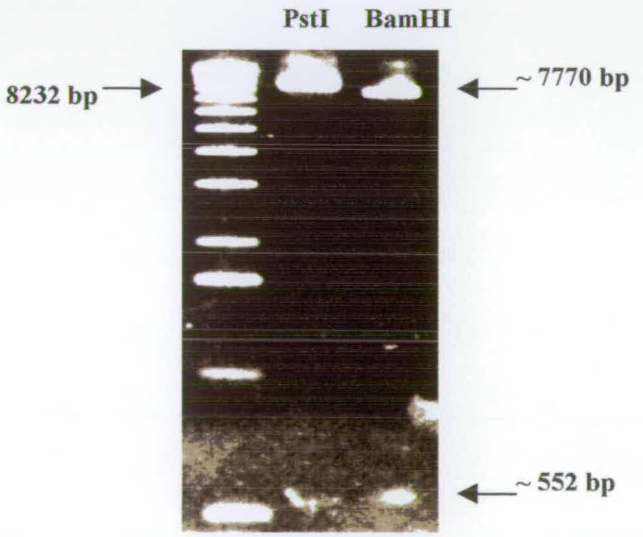
Figure 4.1 Strategy for disruption of *scy*A in *S. frigidimarina* NCIMB400 (see text for details). A - *Acc*I; A^p - *Apa*I; B - *Bam*HI; B^c - *Bcl*I; E - *Eco*RI; E^v - *Eco*RV; H - *Hind*III; K - *Kpn*I; N - N*de*I; P - *Pst*I; S - *Sma*I; Sa - *Sal*I; S^p – *Sph*I;X - *Xba*I.



vector is based on the P15A origin of replication, and includes the *sac*B gene from *Bacillus subtilis*, which is inducible by sucrose and is lethal when expressed in Gram negative bacteria. The *scy*A::ΩSm construct was excised from pAH510 on an *Eco*RI-*Acc*I fragment, blunt-ended with T4 DNA polymerase, then ligated into the *Sma*I site of pJQ200KS (Figure 4.1). The resulting hybrid plasmid was designated pAH512.

Transformants containing pAH512 were selected for by plating on LB supplemented with streptomycin and gentamycin. The region containing the ΩSm cassette was also sequenced to ensure incorporation at the correct position (results not shown). Finally, pAH512 was digested with *Pst*I and *Bam*HI, again to verify the construct (Figure 4.2).

Figure 4.2. Verification of recombinant plasmid pAH512. Plasmid DNA was digested with *Bam*HI or *Pst*I for 3 hours at 37 °C and then separated on a 1.2 % agarose gel. For a physical map of pAH512 see figure 4.1.



As shown in figure 4.2, *Pst*I cuts pAH512 once to give a linear plasmid of 8232 bp. The *Pst*I site adjacent to *Psh*AI (Figure 4.1) is lost during cloning due to overlapping of these sites. The *Bam*HI site adjacent to the *Acc*I site from pTZ18r is also lost during blunt-ending of the *Acc*I site and subsequent cloning into pJQ200KS. *Bam*HI now cuts twice to give fragments of approximately 552 bp and 7770 bp.

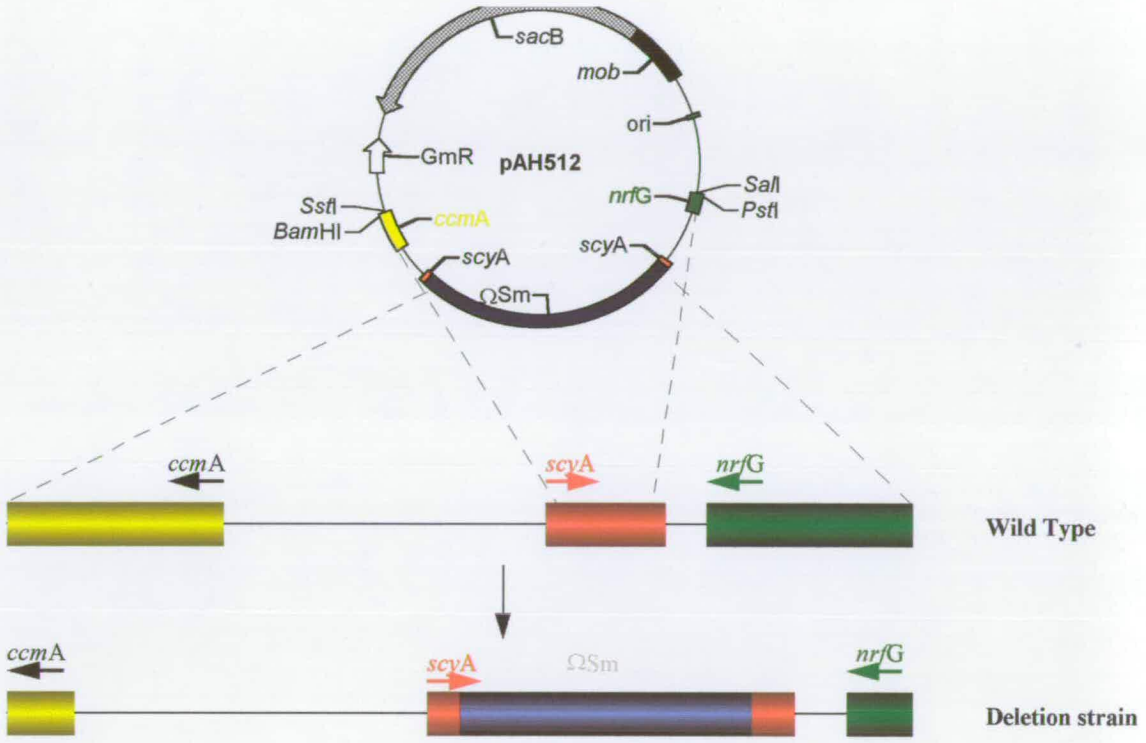
4.1.2 Effecting the cytochrome c_5 deletion

In order to eliminate the chromosomal copy of *scy*A in *Shewanella frigidimarina*, the *scy*A::ΩSm construct had to replace the wild-type gene. This was accomplished as follows. Firstly, in order to introduce pAH512 into *S. frigidimarina*, the plasmid was transformed from *E. coli* strain DH5α, in which it was constructed, to strain SM10. Plasmid pAH512 is *mob*$^+$ and SM10 is a conjugative strain of *E. coli* which may be mated with a variety of Gram negative bacteria. Transformants were again verified by growth on streptomycin and gentamycin- supplemented LB plates.

Plasmid pAH512 was introduced from *E. coli* SM10 into wild type *S. frigidimarina* using filter-mating. Both donor and recipient cells were cultured to exponential phase and mixed at a ratio of approximately 10^6 donor cells and 10^7 recipient cells. Following mating, exconjugant cells were selected for by plating on LB agar supplemented with streptomycin, rifampicin and 5 % sucrose. Streptomycin selects for insertion of the resistance cassette, rifampicin for *S. frigidimarina*, and sucrose against the *sac*B gene of pJQ200KS.

In a typical mating reaction over 300 exconjugant colonies had grown following 3 days incubation at 23 °C. All of the colonies had the expected phenotype of streptomycin, rifampicin and sucrose resistance. No spontaneous mutants of *S. frigidimarina* or *E. coli* were found on LB with the same supplements. These results imply that for the new strain, the streptomycin resistance cassette had been acquired and the suicide vector had been lost along with the associated *sac*B gene, or that both of these markers had been inactivated. The *S. frigidimarina* Sm^r strain arising by double cross-over events should contain an inactive *scy*A gene replacing the wild-type genomic copy (Figure 4.3).

Figure 4.3 . Disruption of *scy*A. Homologous recombination replaces the wild type chromosomal copy of *scy*A with a disrupted copy.



Five colonies from selection plates were picked for further analysis.

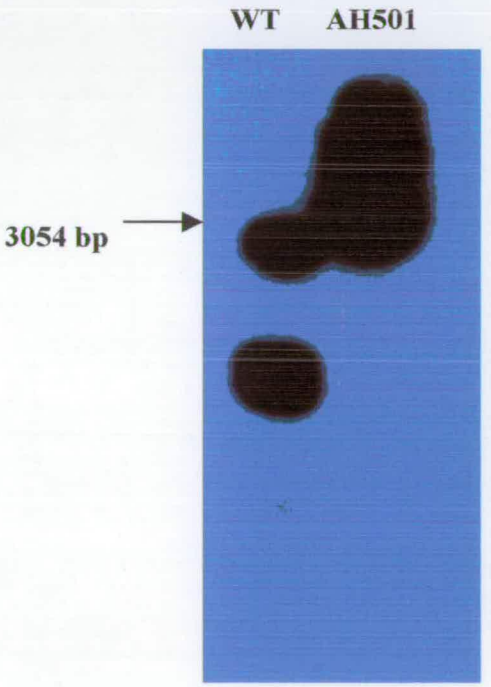
4.1.3 Verification of cytochrome c_5 deletion strain

Southern blotting and haem stain analysis were used to demonstrate that the chromosomal *scy*A gene was exchanged with the inactivated *scy*A gene, and cytochrome c_5 was absent from the deletion strain.

4.1.3.1 Southern blot analysis

Chromosomal DNA was isolated from wild-type and mutant strains and digested with *Pst*I. Following Southern blotting of the separated DNA, a 1089 bp region of PCR amplified wild-type *S. frigidimarina* DNA encompassing the entire cytochrome c_5 gene and surrounding regions was used as a probe. The resulting autoradiograph is shown in figure 4.4.

Figure 4.4. Autoradiograph of a Southern blot of wild-type *S. frigidimarina* and cytochrome c_5 deletion strain, AH501, genomic DNA, digested with *Pst*I and probed with an α^{32}P-labelled PCR fragment corresponding to *S. frigidimarina* cytochrome c_5. The blot was washed in 2x SSC; 0.1 % SDS at room temperature for 15 mins, then at 65 °C for 15 mins before exposure.
WT – wild type.



As shown in figure 4.4, the PCR probe hybridised to two fragments, of approximately 3,000 bp and 1,600 bp, of wild-type *S. frigidimarina*, and a single fragment of approximately 6,000 bp of the deletion strain, AH501. Examination of the restriction sites within *scy*A, shown in figure 4.1, reveals that the *Pst*I site present within the wild-

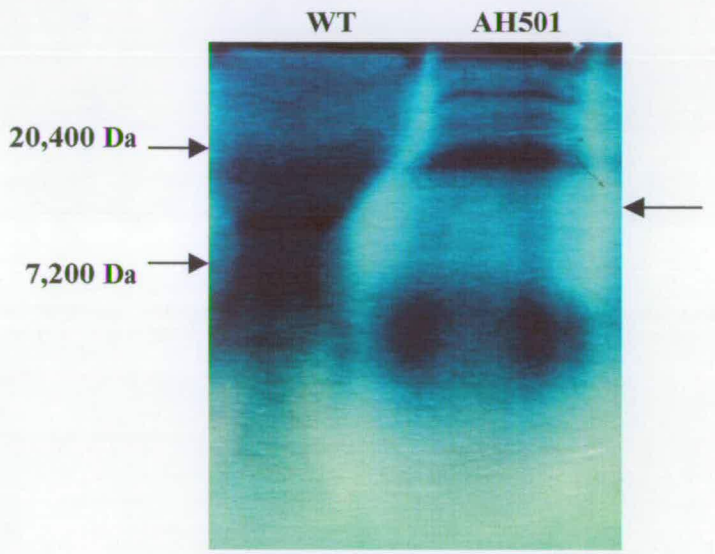
type gene is destroyed upon ligation of the ΩSm cassette into the neighbouring *Psh*AI site. DNA from the deletion strain is therefore not digested with *Pst*I and a single band hybridising to the probe is seen. Wild-type *S. frigidimarina* chromosomal DNA, digested with *Pst*I, shows two bands hybridising to the probe indicating an intact *Pst*I site. These results therefore confirm that the genomic *scy*A gene was exchanged with the inactivated copy.

4.1.3.2 Haem stain analysis

Before phenotypic characterisation of the deletion strain could be carried out, the absence of cytochrome c_5 had to be demonstrated. Due to the number of cytochromes with similar spectral properties found in *S. frigidimarina*, the absence of cytochrome c_5 can not be shown using spectral analysis. However, the presence of covalently bound haem within the cytochromes *c* enables the use of haem-stain analysis.

To this end, soluble fractions of microaerobically grown wild-type and deletion strains were isolated and fractionated by SDS PAGE. Following fractionation, gels were stained for haem as described in chapter 2. Results are given in figure 4.5.

Figure 4.5. Analysis of *c*-type cytochromes in soluble fractions of wild-type- and cytochrome c_5 deletion strains of *S. frigidimarina* by SDS-PAGE and haem staining. Soluble fractions of wild-type and the cytochrome c5 deletion strain were fractionated through a 15 % SDS-PAGE gel, then stained for haem. Biorad Broad range prestained markers were used as size standards.



As shown in figure 4.5, a band corresponding to the appropriate size for cytochrome c_5, present in the wild-type strain, is missing in the deletion strain. The amounts of the other cytochromes were not affected by the mutagenesis of cytochrome c_5.

The results described above show that the structural gene, *scy*A, has been disrupted, and the resulting deletion strain lacks cytochrome c_5.

4.2 Phenotypic characterisation

4.2.1 Characterisation of the overall anaerobic respiratory capability of *Shewanella frigidimarina* NCIMB400

S. frigidimarina NCIMB400, like many other members of this genus, displays remarkable respiratory versatility as it is able to respire on a variety of compounds as sole terminal electron acceptors. To facilitate characterisation of the mutant strain described above, a suitable assay system had to be generated. This was achieved using solid minimal medium agar plates supplemented with a variety of electron donors and acceptors. Several different media were initially tested to ensure that bacterial growth was due solely to the given supplements (for recipes see chapter 2). With these supplements growth is entirely dependent on anaerobic respiration since neither the donors nor the acceptors can be fermented by *S. frigidimarina*. Single colonies from overnight LB cultures of the appropriate strain were used to inoculate each minimal medium plate and incubated under a H_2/CO_2 atmosphere. Each donor, acceptor, and donor + acceptor combination was tested in sextuplicate. Results indicative of growth were the formation of colonies following 6 to 8 weeks incubation, as shown in figure 4.6.

Growth on minimal media plates was confirmed as *Shewanella* by subculturing. Tabulated results for characterisation of wild type *S. frigidimarina* are given in table 4.1

Figure 4.6. Evidence of growth on minimal medium plates. Minimal medium plates were inoculated with an overnight culture of the appropriate strain and incubated under a H_2/CO_2 atmosphere. Following 6-8 weeks plates were scored for growth. Sections 2-6 would be scored "✓", whereas section 1 would be described as "×".

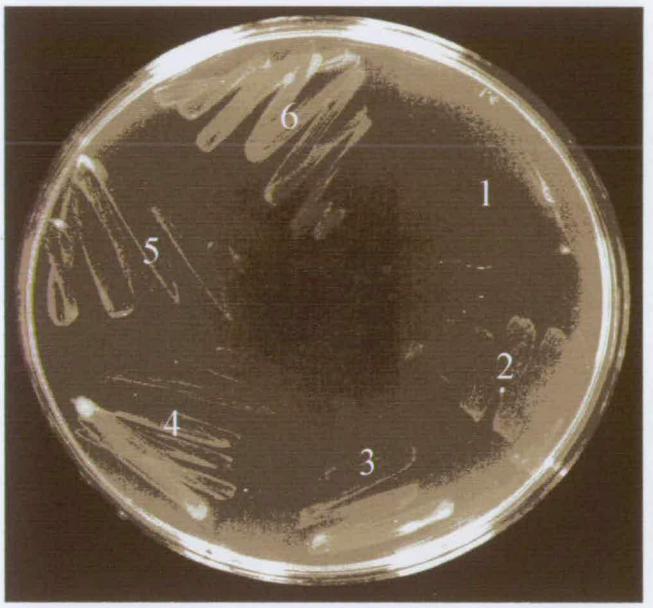


Table 4.1 Growth of wild type *Shewanella frigidimarina* NCIMB400. The organic carbon sources were all at 15 mM. Each electron acceptor concentration is given in chapter 2. Growth was assessed for each combination in sextuplicate following 6-8 weeks of anaerobic growth at room temperature.

	Electron donor				
Electron Acceptor	**Acetate**	**Formate**	**Lactate**	**Pyruvate**	**Succinate**
Oxygen	×	✓	✓	✓	✓
Iron (iii) citrate	✓	×	✓	×	×
Iron sulphate	×	✓	✓	×	×
Potassium nitrate	✓	✓	×	×	✓
Sodium nitrate	×	✓	×	×	✓
Ammonium sulphate	×	×	×	×	×
Manganese	×	×	×	×	×
Nitrite	×	×	×	✓	✓
DMSO	×	✓	✓	×	×
TMAO	×	×	×	✓	✓
Fumarate	✓	✓	✓	✓	×
Tetrathionate	✓	×	×	✓	✓
Sulphite	✓	×	×	✓	✓
Glycine	✓	×	×	✓	✓
None	×	×	×	×	×

As shown in the above table, specific coupling between the organic carbon substrate and the terminal electron acceptor was observed in growth experiments with *S. frigidimarina.* Wild type *S. frigidimarina* was capable of growth on:

(i) acetate and either Fe^{3+} (as iron III citrate), NO_3^- (as KNO_3), fumarate, tetrathionate, sulphite, or glycine;

(ii) formate and either O_2, Fe^{3+} (as iron III sulphate), NO_3^-, DMSO or fumarate;

(iii) lactate and either O_2, Fe^{3+}, DMSO or fumarate;

(iv) pyruvate and either O_2, NO_2^-, TMAO, fumarate, tetrathionate, sulphite, or glycine;

(v) succinate and either O_2, NO_3^-, NO_2^-, TMAO, tetrathionate, sulphite, or glycine.

Surprisingly, no growth was observed for wild-type under aerobic conditions with acetate as organic carbon source. It seems possible that the incubation temperature increased above the limit for growth of *Shewanella.* However, the cultures were tested in sextuplicate, therefore it seems unlikely that identical results should be obtained in each case.

S. frigidimarina was unable to form colonies on minimal media supplemented with each carbon source alone, each electron acceptor alone, or on unsupplemented media, under anaerobic conditions (Table 4.1).

These results were also obtained following growth in liquid minimal media incubated at 23 °C for one week, using the same supplements. Medium was added to completely fill the bottles, then tightly sealed. Growth was assessed by reading the optical density of media at 500 nm, using uninoculated medium as a blank.

4.2.2 Phenotype of a cytochrome c_5 deletion strain

The plate assay system developed above was used to characterise the anaerobic respiratory capability of AH501, the cytochrome c_5 deletion strain. Results are given in table 4.2. Three mutant strains from different exconjugant plates were tested in duplicate, along with wild type *S. frigidimarina* NCIMB400.

Table 4.2 Growth of a *Shewanella frigidimarina* NCIMB400 cytochrome c_5 deletion strain, AH501. The organic carbon sources were all at 15 mM. Each electron acceptor concentration is given in chapter 2. Growth was assessed for each combination in sextuplicate following 6-8 weeks of anaerobic growth at room temperature.

	Electron donor				
Electron Acceptor	**Acetate**	**Formate**	**Lactate**	**Pyruvate**	**Succinate**
Oxygen	✗	✓	✓	✓	✓
Iron (iii) citrate	✓	✗	✓	✗	✗
Iron sulphate	✗	✗	✓	✗	✗
Potassium nitrate	✓	✗	✗	✗	✗
Sodium nitrate	✗	✗	✗	✗	✗
Ammonium sulphate	✗	✗	✗	✗	✗
Manganese	✗	✗	✓	✓	✗
Nitrite	✗	✗	✗	✓	✓
DMSO	✗	✗	✓	✗	✓
TMAO	✗	✓	✗	✓	✓
Fumarate	✓	✓	✓	✓	✗
Tetrathionate	✓	✗	✓	✓	✗
Sulphite	✓	✗	✗	✓	✗
Glycine	✓	✗	✗	✓	✓
None	✗	✗	✗	✗	✗

AH501 exhibited similar anaerobic respiratory capacity with respect to wild type, the most notable exception being the lack of coupling between formate or succinate oxidation and nitrate reduction (Table 4.2).

4.3 Discussion

A *Shewanella frigidimarina* NCIMB400 cytochrome c_5 deficient mutant has been constructed via homologous recombination between the chromosomal *scy*A gene and a defective *scy*A gene on a suicide plasmid. The deletion strain was then verified by Southern blotting and haem stain analysis.

The physiological function of cytochrome c_5 was assessed by testing the ability of the deletion strain to grow anaerobically using a variety of electron donors and acceptors. The mutant strain shares identical anaerobic respiratory capacity with respect to wild type, with the exception of coupling between formate oxidation and nitrate reduction.

Historically, cytochromes c_5 have been linked with cytochromes c_4 due to their copurification (Haddock and Jones, 1977). Both of these proteins are small, single-haem cytochromes, which have positive redox potentials.

The majority of information on both cytochrome c_4 and c_5 has come from studies of *Azotobacter*. *Azotobacter* are obligate aerobes, capable of fixing atmospheric nitrogen. They possess the highest cellular respiratory rate of any known organism. As a result of this, examination of their respiratory networks has attracted much attention.
A tentative branched scheme of *Azotobacter* respiratory chains was first presented in 1967 by Jones and Redfearn. Subsequent investigation resulted in a proposal that two separate terminal electron transport pathways exist: a major electron flow catalysed by cytochromes b_1 and a_2, and a minor flow catalysed by cytochromes c_4 and c_5 and $a_1$0 (Ackrell and Jones, 1971). Jones further proposed that cytochromes c_4 and c_5 contribute to the phenomenon of nitrogenase protection by acting in a sequential pathway passing electrons to a cytochrome oxidase (Ackrell, Erickson, and Jones, 1972; Haddock and Jones, 1977).

Since then, evidence has been provided to suggest that cytochromes c_4 and c_5 operate in parallel pathways rather than in a single pathway. These include significantly different

P/O ratios when used as electron mediators between ascorbate and the respiratory electron transport chain (Jurtshuk and Yang,1980). Such enzymatic studies have now been supplemented by the use of deletion strains. Recent experiments using mutants in cytochrome c_4 have also shown that in *Azotobacter*, cytochrome c_4 and c_5 act in separate electron flow pathways both involving cytochrome *o* as the terminal oxidase (Ng *et al.*, 1995). Mutants lacking cytochrome c_4 have normal respiratory ability with physiological substrates but have greatly reduced levels of ascorbate-TMPD oxidation rate. The new separate but parallel *c*-type electron flow pathway may explain the lack of growth differences which was found between the cytochrome c_4 mutants and wild-type. A mutant in cytochrome *o* was also found to be identical to the cytochrome c_4 mutant (Ng *et al.*, 1995).

More recently, mutants in *Azotobacter* cytochrome c_5 have been constructed (Rey and Maier, 1997). Mutants in this cytochrome as well as mutants in both cytochrome c_4 and c_5 were characterised and the roles of putative environmental regulatory factors, oxygen and nitrogen, were examined. The individual cytochrome c_4 and c_5 mutants were slightly deficient in growth with respect to wild-type at low O_2 tensions, whereas the double mutant was more noticeably retarded at the lower O_2 levels. In other words, the growth deficiency of the double mutant is especially apparent when the energy and respiratory demands are great. The conclusion derived from these experiments is that the role of the cytochrome *c*-dependent pathways is to augment respiration in intermediate and low O_2 environments.

LacZ protein fusions with both cytochromes c_4 and c_5 were also constructed by Rey and Maier (1997). Growth of strains harbouring each fusion construct in media with a nitrogen source produced cells with a significantly lower cytochrome c_5- *lac*Z fusion activity than cells cultured in media lacking nitrogen. Both cytochrome c_4 and c_5 are up-regulated upon nitrogen-starvation, therefore c_5o pathway expression may be highly correlated with nitrogen fixation.. The up-regulation of cytochromes c_4 and c_5 could be due to known regulatory elements that act on *nif* genes such as *nif*A and *ntr*A. These factors have been shown to affect the up-regulation of genes ancillary to nitrogen

fixation. Alternatively, up-regulation may be due to a factor like the *Klebsiella* nitrogen assimilation control protein that couples transcription of σ^{70}-dependent promotors to regulation by the nitrogen regulatory system (Rey and Maier, 1997; Bender, 1991). Indeed two putative σ^{N} promotors may be found upstream of *scy*A (Table 3.2).

Unlike *Azotobacter*, *Shewanella* do not fix nitrogen, but they are facultative anaerobes, as opposed to obligate aerobes. Such up-regulation by nitrogen would not therefore be expected with *Shewanella*. However a role as a component of a respiratory chain to cytochrome oxidase would seem a reasonable proposition. It may seem plausible that cytochrome c_5 acts to optimise the energy yield from respiration in this obligately respiratory organism, by acting as a donor to terminal oxidase(s) bridging the gap between changes from aerobic to anaerobic respiration and vice versa. In line with this hypothesis, Northern analysis of *Shewanella* cytochrome c_5 in chapter 3 showed that expression of this cytochrome is constitutive.

The results described in this chapter suggest that cytochrome c_5 from *Shewanella frigidimarina* NCIMB400 may have a role in respiration with nitrate as the terminal electron acceptor. *Shewanella* have previously been shown to reduce nitrate; nitrate is the preferred terminal electron acceptor under anaerobic conditions. Indeed, *S. putrefaciens* has the unusual ability to respire nitrate under aerobic conditions (Krause and Nealson,1997). In addition, *c*-type cytochromes have been implicated in respiratory chains involving nitrate. An example is provided by *Rhodobacter sphaeroides*; two types of nitrate reductase have been isolated and characterised from this organism, one of which is a periplasmic enzyme associated with a c_{552} cytochrome (Castillo *et al.*,1996; Richardson *et al.*, 1990).

The nitrate reductases characterised from *Shewanella* are membrane-bound, highly sensitive to oxygen and positively regulated by the presence of substrate. Cytochrome c_5 could be involved in respiratory protection of nitrate reductase, if it is not directly involved in donating electrons to it.

Shewanella are also known denitrifying bacteria (Krause and Nealson,1997). *Shewanella putrefaciens* dissimilates nitrate to ammonium in media with glucose and a high redox potential (Samuelson *et al.*, 1995).

The region encoding cytochrome c_5, described in chapter 3, is found to contain two other operons, one of which is *nrf* (for, *n*itrate *r*eduction by *f*ormate) which is involved in cytochrome *c* biogenesis (Saffarini *et al.*, 1998). The other encodes genes involved in *c*ytochrome *c* *m*aturation (*ccm;* see chapter 1, section 1.7). The *aeg*46.5 operon of *E. coli* also encodes *nrf* and *ccm* genes. In addition, cytochromes involved in electron transfer from quinones to a periplasmic nitrate reductase are encoded in this region in *E. coli* (Grove *et al.*,1996). These cytochromes, which show little sequence similarity to cytochromes c_5, are as yet uncharacterised.

If we consider the organisation of respiratory chains, the oxidation-reduction potentials of the compounds that constitute electron transport chains are poised such that each component can be reduced by the reduced form of the preceding component. The midpoint potential *of S. frigidimarina* NCIMB400 cytochrome c_5 was found to be + 274 mV (Allen, unpublished). If we compare this value with potential electron donors and acceptors for cytochrome c_5, we can see that cytochrome c_5 may be reduced by any of the potential electron donors shown in table 4.3. In turn the midpoint potential of cytochrome c_5 is such that this protein is theoretically capable of reducing nitrite, nitrate, iron, or oxygen (Table 4.3).

Table 4.3 Midpoint potentials of putative electron donors and acceptors used in characterisation of cytochrome c_5 from *Shewanella frigidimarina* NCIMB400.

Electron donor	Midpoint potential (mV)	Cytochrome c_5	Electron acceptor	Midpoint potential (mV)
Acetate	- 659	+ 274 mV	Oxygen	+ 820
Formate	- 432		Fe (III)	+ 770
Lactate	- 197		Nitrate	+ 420
Pyruvate	-197		Mn(IV)	+ 380
Succinate	+30		Nitrite	+ 360
			DMSO	+160
			TMAO	+130
			S^0	- 240
			Thiosulphate	- 372
			Sulphite	- 516

Cytochrome c_5 would not be able to pass electrons to dimethylsulphoxide, trimethylamine oxide, elemental sulphur, thiosulphate or sulphite, all of which are reduced at potentials much lower than + 274 mV. Indeed, the ability of *S. frigidimarina* NCIMB400 to couple respiration with these electron acceptors was unaffected by deletion of cytochrome c_5.

A role in electron transfer to terminal oxidase(s) is not the only function described for cytochrome c_5. Recently, Petushkov and Lee (1997) isolated two small cytochromes from *Vibrio fischeri.* These cytochromes share a number of features with cytochromes c_4 and c_5. *Vibrio fischeri* is a bioluminescent organism, and the role of these cytochromes is considered as mediating a two-electron process relevant to the bioluminescence function.

Appia-Ayme *et al.* (1998) recently sequenced the *cyc*1 gene encoding *Thiobacillus ferrooxidans* c_{552} cytochrome. This protein is a 21 kDa periplasmic c_4-type cytochrome. Reduction kinetics experiments indicated that this cytochrome participates in a respiratory chain linking rusticyanin to a terminal cytochrome *c* oxidase. Analysis of the region encoding this cytochrome reveals that *cyc*1 is co-transcribed with another gene, *cyc*2, which encodes an atypical periplasmic protein with a molecular weight of 49.7

kDa. The function of this protein is unknown, but its cosynthesis with cytochrome c_4 suggests that these proteins are functionally related (Appia-Ayme *et al.*, 1998).

The use of deletion strains to characterise protein function has been widely used in examination of *c*-type cytochromes. This method is not without its disadvantages, as described.

Tulley *et al.* (1991) used a deletion strain of *Bradyrhizobium japonicum* to determine the physiological role of one of two small cytochromes, cytochrome c_{555}. These two cytochromes, c_{550} and c_{555}, have midpoint potentials of +277 mV and +236 mV, respectively. They appear to be small, single-haem cytochromes, which have positive midpoint potentials and function as electron donors to a terminal oxidase. Expression of cytochrome c_{550} is found to increase under anaerobic or semianaerobic conditions in a variety of strains. Following deletion of cytochrome c_{555}, no differences were noted between the mutant strain and wild-type following an array of growth experiments and extensive biochemical characterisation. This lack of mutant phenotype with c_{555} mutant was attributed to an ability of cytochrome c_{550} to compensate for the deleted protein – it is possible that c_{555} and c_{550} may be equally available for a variety of reactions that generate reducing equivalents. Future work to help identify the physiological role will involve multiple knockouts.

Similarily, Stoll *et al.* (1996) examined the function of cytochrome c_{550} in *Paracoccus denitrificans*. Again, deletion of the cytochrome from the chromosome resulted in no mutant phenotype and the function is somewhat of an enigma. Cytochrome c_{550} expression has often been regarded as constitutive although *LacZ* fusions studied by these authors suggest it is induced under denitrifying growth conditions. Compensation for this cytochrome by another protein was used to explain the lack of mutant phenotype.

From the limited knowledge already accumulated, the earlier suppositions which indicated the high degree of complexity of bacterial electron transport systems have proven true when compared to those of mitochondria. The ability of bacteria to use several different proteins for the same function may be contributory to their respiratory flexibility.

To demonstrate that the growth phenotype of AH501 strain using nitrate as an electron acceptor is indeed due to disruption of *scy*A and the absence of cytochrome c_5, rather than some undefined secondary mutation or effect, complementation of the cytochrome using pAH512 may be attempted. Use of *Lac*Z fusions would also facilitate the detection of changes in expression of cytochrome c_5 under a variety of growth conditions.

However, in order to elucidate the physiological role of cytochrome c_5 in *Shewanella frigidimarina* NCIMB400 it is imperative that multiple cytochrome knockout strains are produced, in particular deletion of cytochrome c_4.

Chapter 5

Cytochrome c_3 from *Shewanella frigidimarina* NCIMB400: Gene Disruption and Phenotypic Characterisation

Cytochromes c_3 have been the subject of extensive investigations for over three decades, resulting in a wealth of both biochemical and structural data. They are small, tetrahaem proteins of low redox potential, and are characteristic of the sulphate reducing bacteria of the genus *Desulfovibrio*.

A small, acidic, low potential tetrahaem cytochrome with similarities to the cytochromes c_3 from sulphate-reducing bacteria, has been isolated from the periplasm of *Shewanella frigidimarina* NCIMB400. *Shewanella* are the first organisms capable of aerobic growth that have been found to contain this protein and its presence raises interesting questions about the architecture of the respiratory chains of this species. In addition, *Shewanella species* are the only known facultative anaerobes also capable of dissimilatory growth with elemental sulphur as an electron acceptor.

Due to the appearance of cytochromes c_3 almost exclusively within organisms of the genus *Desulfovibrio*, their proposed function has been one of participation in one or more pathways of sulphur metabolism. They are also thought to act as the natural electron donor for periplasmic hydrogenase (see chapter 1). However, genetic evidence for the role of cytochromes c_3 is lacking.

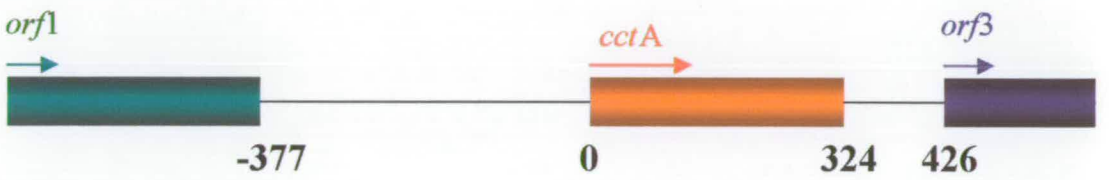
Work in this chapter describes the approach taken to identify the physiological electron donor(s) and acceptor(s) of cytochrome c_3 from *S. frigidimarina* NCIMB400, the results obtained and the implications they have on our understanding of the metabolic pathways within this organism.

5.1.1 Cloning and sequencing of cytochrome c_3

A fragment containing the cytochrome c_3 structural gene and the surrounding region of *S. frigidimarina* genomic DNA has been cloned and sequenced (Gordon *et al.*, unpublished).

The structural gene coding for cytochrome c_3 (*cct*A) is 324 bp long, encoding a protein with a putative signal sequence of 25 amino acids and a mature polypeptide of 83 residues (Figure 5.1).

Figure 5.1. Organisation of the region encoding the cytochrome c_3 structural gene, *cct*A, in *Shewanella frigidimarina* NCIMB400. Arrows indicate direction of transcription.



Immediately downstream of the translational stop codon a region with similarity to bacterial *rho*-independent transcriptional terminators is found, implying that the *cct*A gene is not co-transcribed with any other coding sequence. In addition, *cct*A is flanked by coding sequences (in different reading frames) that are clearly unrelated to anaerobic respiration (Figure 5.1). Sequencing downstream of the *cct*A gene revealed a coding sequence in the same orientation to *cct*A which encodes a putative protein product with extensive similarity to nitrate and formate reductases (*orf*3; Figure 5.1). These are cytoplasmic enzymes involved in the utilisation of nitrate as a nitrogen source. Upstream of *cct*A is a further reading frame in the same orientation (*orf*1; Figure 5.1). This shows extensive similarity to 3- hydroxyisobutyrate dehydrogenases which are again cytoplasmic enzymes in bacteria involved in valine catabolism. These sequences may be found in the EMBL database entry (AJ000006).

That *cct*A is not cotranscribed with any other coding sequence was also demonstrated through Northern blot analysis; the *cct*A mRNA is in the region of 400-600 bp in length (Gordon, unpublished).

Sequencing of the region surrounding the structural gene has not revealed any proteins which may be functionally related to *S. frigidimarina* NCIMB400 cytochrome c_3.

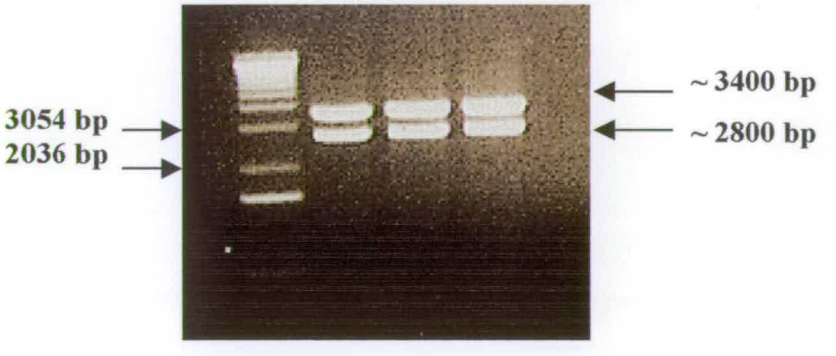
Construction of mutants is an essential first step in determining function. It has been shown by gene disruption that flavocytochrome c_3 (fcc_3) is clearly required for fumarate reduction but not for other electron transfer pathways (Gordon *et al.*, 1998). A similar approach will help identify the physiological role of cytochrome c_3.

5.1.2 Targeted disruption of cytochrome c_3 in *S. frigidimarina* NCIMB400

An approximately 1700 bp fragment encompassing the entire cytochrome c_3 structural gene and the 3' and 5' non-coding regions was used to direct the specific integration of the pHRP310 streptomycin/spectinomycin resistance gene into the homologous region of the *Shewanella* genome.

The initial construct for this purpose was generated by Euan Gordon (unpublished work). The 1700 bp region of *S. frigidimarina* DNA containing *cct*A was cloned into pK18, digested with *Bsa*I and *Bst*XI to remove the entire *cct*A coding sequence, and the ends blunt-ended with T4 DNA Polymerase. Into this site a spectinomycin/streptomycin resistance cassette, obtained from pHRP310 on a *Hind*III fragment, was blunt-end cloned, to give pEG801.

Figure 5.2. Agarose gel electrophoresis of plasmid pEG801 following digestion with *Eco*RI and *Hind*III for 3 hours at 37 °C. Gibco-BRL 1 kb Markers used as size standards.



Recombinant plasmid pEG801 was verified by digestion with *Eco*RI and *Hind*III (Figure 5.2). Digestion with these endonucleases releases an approximately 2,800 bp

fragment corresponding to pK18, and an approximately 3,400 bp fragment corresponding to the disruption construct *cct*A::ΩSm.

In order to effect the cytochrome c_3 deletion, the *cct*A::ΩSm deletion construct was transferred from pEG801 to the suicide vector pJQ200KS on a *Sac*I/*Pst*I fragment (map of pJQ200KS shown in figure 4.1, page 121). The resulting plasmid was named pEG720.

Work in this chapter describes the introduction of the deletion construct into both wild type *Shewanella frigidimarina* NCIMB400 and into the fcc_3 deletion strain of *S. frigidimarina* NCIMB400, named EG301, and phenotypic characterisation of the resultant mutants.

5.1.3 Effecting the cytochrome c_3 deletion

S. frigidimarina cytochrome c_3 deletion mutants were constructed by conjugation with a mobilising strain of *E. coli*, SM10, harbouring the *cct*A::ΩSm construct on a suicide vector, pJQ200KS (pEG720). This mutagenesis technique relies on the fact that pJQ200KS acts as a suicide plasmid in *S. frigidimarina* since its replication origin cannot function in this bacterium.

Two recipient *Shewanella frigidimarina* strains were used in mating experiments. The first recipient was wild type *S. frigidimarina* NCIMB400; the second was a strain of *S. frigidimarina*, EG301, in which the flavocytochrome c_3 (fcc_3) gene has been disrupted.

Filter matings were conducted on LB plates at 23 °C overnight using an input cell ratio of approximately 10^7 exponential phase recipient cells and 10^6 exponential phase donors.

Conjugal mating resulted in over 200 exconjugant colonies after incubation for 3 days at 23 °C on LB plates supplemented with streptomycin, rifampicin and 5% sucrose (selection for *S. frigidimarina* NCIMB400) or streptomycin, kanamycin and 5% sucrose (selection for *S. frigidimarina* EG301).

All of the colonies had the expected phenotype of streptomycin, rifampicin (or kanamycin), and sucrose resistance. Spontaneous *S. frigidimarina* NCIMB400 or EG301 mutants were not found on LB with the same supplements. These results imply that for both new strains, the streptomycin resistance cassette had been acquired, and the suicide vector had been lost along with the associated *sac*B gene, or that both of the latter markers had been inactivated. *S. frigidimarina* Sm^R strains arising by double-crossover events should contain an inactive *cct*A operon replacing the wild-type genomic copy.

Ten colonies for each cytochrome c_3 deletion strain were picked for further analysis, AH301-AH310 (NCIMB400 Δc_3), and AH401-AH410 (EG301 Δc_3).

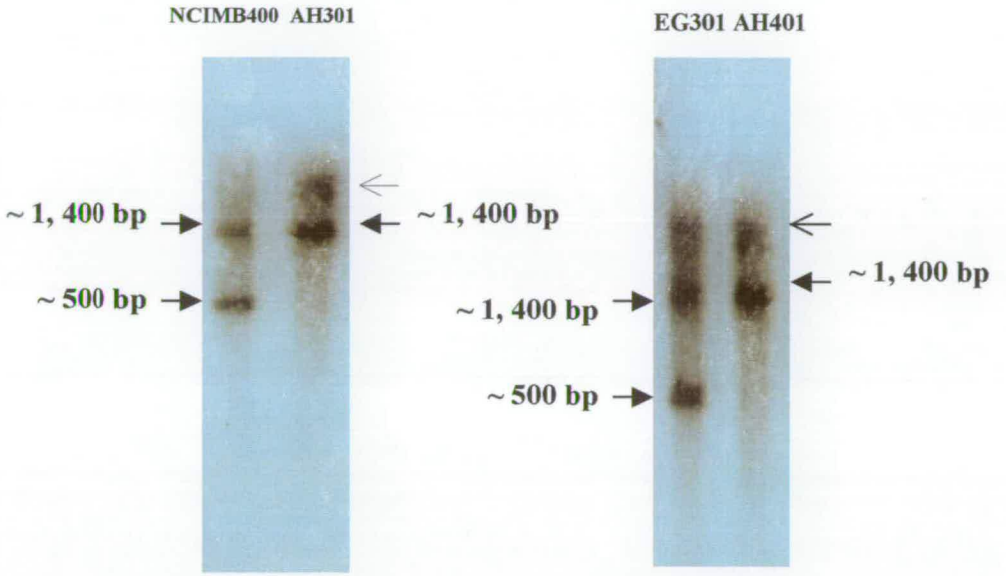
5.1.4 Verification of cytochrome c_3 deletion strains

Southern blotting and tris-tricine SDS-PAGE gel analysis were used to demonstrate that: (1) the genomic *cct*A gene was exchanged with the inactivated gene, and (2) cytochrome c_3 was absent from the deletion strains.

5.1.4.1 Southern blot analysis

Chromosomal DNA was isolated from wild-type and mutant strains and digested with *Pst*I. Following Southern blotting of the separated DNA, pEG720, which contains the disrupted *cct*A gene on a suicide plasmid, was used as a probe. The resulting autoradiograph is shown in figure 5.3.

Figure 5.3 Autoradiograph of a Southern blot of genomic DNA extracted from *S. frigidimarina* NCIMB400, EG301, AH301(Δc_3) and AH401(Δc_3;Δfcc_3), digested with *Pst*I, then probed with $^{[32]}$P-labelled pEG720. The blot was washed in 2x SSC; 0.1 % SDS at room temperature for 15 mins, then at 65 °C for 15 mins before exposure.



As shown in figure 5.3, the probe, pEG720, hybridises to two fragments of approximately 500 bp and 1, 400 bp, in the wild type strains, and only one in the deletion strains (~1,400 bp). The higher molecular weight bands indicated by a thin arrow correspond to unspecific hybridisation during reprobing with molecular weight markers. Analysis of the sequence for the structural gene shows that a *Pst*I site is encoded within the sequence removed during construction of the deletion strain (Gordon, unpublished results). Therefore, genomic DNA from the deletion strains is not cut at this position whereas wild type genomic DNA is and subsequently only one hybridising band is observed.

5.1.4.2 Tris-Tricine SDS PAGE gel analysis

Southern blot hybridisation has shown that *cct*A has been disrupted in the mutant strains (5.1.4.1). In order to demonstrate that the *cct*A mRNA is translated into a cytochrome *c* with the expected size, cellular fractions of AH301 and AH401 were examined.

Use of standard SDS PAGE gels for examining cytochrome c_3 often results in anomalous migration of purified protein. For this reason Tris-Tricine SDS-PAGE gels were employed. Tris-Tricine gels are often used to examine low molecular weight proteins.

To this end, soluble fractions of anaerobically grown wild-type and deletion strains were isolated and fractionated. Following electrophoresis, gels were stained with Coomassie brilliant blue, as for standard SDS-PAGE gels. Results are given in figure 5.4.

Figure 5.4. Analysis of *c*-type cytochromes in soluble fractions of wild-type and cytochrome c_3 deletion strains of *S. frigidimarina* by Tris-Tricine SDS-PAGE (16 %). Biorad Prestained Broad range molecular weight markers were used as size standards.
A. NCIMB400
B. EG301

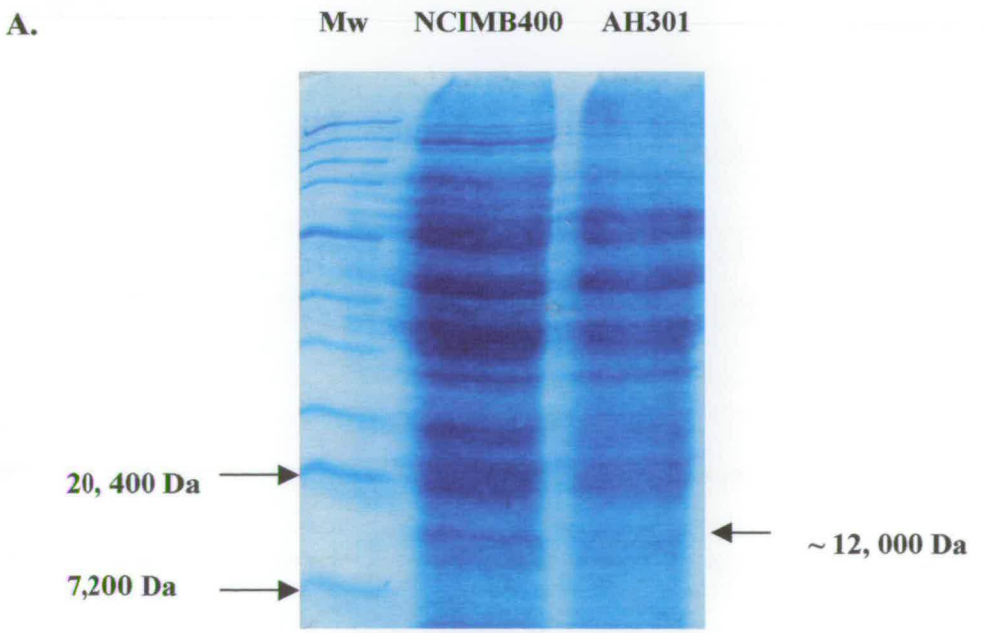
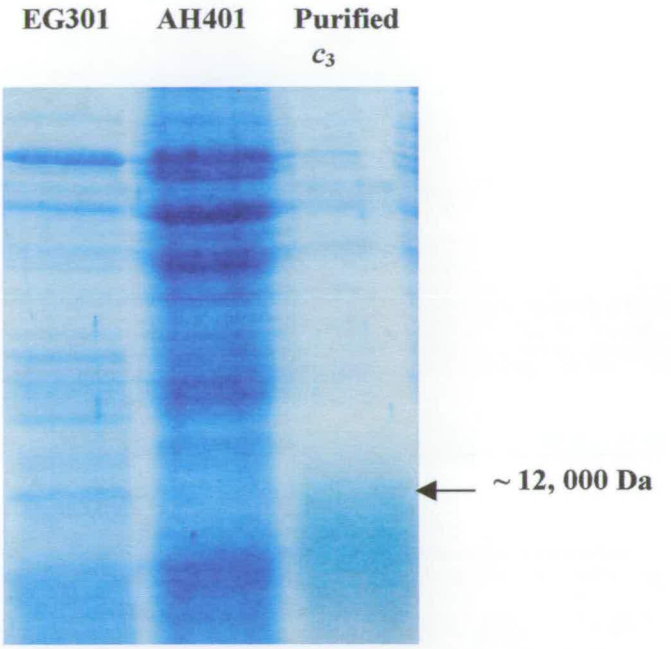


Figure 5.4B.



As shown in figure 5.4, a band corresponding to the appropriate size for cytochrome c_3, present in the wild-type strains, is missing in the deletion strains. The amounts of the other proteins were not affected by the mutagenesis of cytochrome c_3.

5.2 Phenotypic characterisation of cytochrome c_3 deletion strains

5.2.1 Plate assays

A plate assay system was used to characterise the anaerobic respiratory capabilities of AH301 and AH401, the cytochrome c_3 deletion strains. Minimal medium plates supplemented with a variety of electron donors and acceptors, as described in 4.2., were inoculated with the appropriate strains. Results are given in tables 5.1 – 5.5. Three different colonies from different exconjugant plates of each mutant strain were tested in duplicate, along with wild type *S. frigidimarina* NCIMB400 and EG301, the fcc_3 deletion strain. Plates were incubated under a H_2/CO_2 atmosphere at 23 °C. The ability to form colonies following 6-8 weeks incubation was then scored.

Table 5.1 Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 using **acetate** as the carbon source. Results are "best of six" (i.e. 1-3 out of 6 equals no growth whereas 4-6 out of 6 equals growth), scored for growth based on the method described in 4.2.1.

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 (Δfcc_3;Δc_3)**
Oxygen	✗	✗	✗	✗
Iron (III) citrate	✓	✗	✓	✗
Iron sulphate	✗	✗	✗	✗
Potassium nitrate	✓	✓	✓	✗
Sodium nitrate	✗	✗	✗	✗
Ammonium sulphate	✗	✗	✗	✗
MnO_2	✗	✗	✗	✗
Nitrite	✗	✗	✗	✗
DMSO	✗	✗	✗	✗
TMAO	✗	✗	✗	✗
Fumarate	✓	✗	✗	✗
Tetrathionate	✓	✓	✓	✓
Sulphite	✓	✓	✓	✓
Glycine	✓	✓	✓	✓
None	✗	✗	✗	✗

Table 5.2 Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 using **formate** as the carbon source. Results are "best of six", scored for growth based on the method described in 4.2.1.

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 (Δfcc_3;Δc_3)**
Oxygen	✓	✓	✓	✓
Iron (III) citrate	×	×	×	×
Iron sulphate	✓	✓	✓	×
Potassium nitrate	✓	×	✓	×
Sodium nitrate	✓	×	✓	×
Ammonium sulphate	×	×	×	×
MnO_2	×	✓	×	×
Nitrite	×	×	×	×
DMSO	✓	×	✓	×
TMAO	×	×	×	×
Fumarate	✓	×	×	×
Tetrathionate	×	×	×	×
Sulphite	×	×	×	×
Glycine	×	×	×	×
None	×	×	×	×

Table 5.3 Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 using **lactate** as the carbon source. Results are "best of six", scored for growth based on the method described in 4.2.1.

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 (Δfcc_3;Δc_3)**
Oxygen	✓	✓	✓	✓
Iron (III) citrate	✓	×	✓	×
Iron sulphate	✓	×	✓	×
Potassium nitrate	×	×	×	×
Sodium nitrate	×	×	×	×
Ammonium sulphate	×	×	×	×
MnO_2	×	×	×	×
Nitrite	×	×	×	×
DMSO	✓	×	✓	×
TMAO	×	×	×	×
Fumarate	✓	×	×	×
Tetrathionate	×	×	×	×
Sulphite	×	×	×	×
Glycine	×	×	×	×
None	×	×	×	×

Table 5.4. Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 using **pyruvate** as the carbon source. Results are "best of six", scored for growth based on the method described in 4.2.1.

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 (Δfcc_3;Δc_3)**
Oxygen	✓	✓	✓	✓
Iron (III) citrate	×	×	×	×
Iron sulphate	×	×	×	×
Potassium nitrate	×	×	×	×
Sodium nitrate	×	×	×	×
Ammonium sulphate	×	×	×	×
MnO_2	×	×	×	×
Nitrite	✓	✓	✓	✓
DMSO	×	×	×	×
TMAO	✓	✓	✓	✓
Fumarate	✓	✓	×	×
Tetrathionate	✓	✓	✓	✓
Sulphite	✓	×	✓	✓
Glycine	✓	✓	✓	✓
None	×	×	×	×

Table 5.5 Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 using **succinate** as the carbon source. Results are "best of six", scored for growth based on the method described in 4.2.1.

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 (Δfcc_3;Δc_3)**
Oxygen	✓	✓	✓	✓
Iron (III) citrate	×	×	×	×
Iron sulphate	×	×	×	×
Potassium nitrate	✓	✓	✓	✓
Sodium nitrate	✓	✓	✓	✓
Ammonium sulphate	×	×	×	×
MnO_2	×	×	×	×
Nitrite	✓	×	✓	×
DMSO	×	×	×	×
TMAO	✓	✓	✓	✓
Fumarate	×	×	×	×
Tetrathionate	✓	✓	✓	✓
Sulphite	✓	✓	✓	✓
Glycine	✓	×	✓	✓
None	×	×	×	×

As shown in the above tables, specific coupling between the organic carbon substrate and the terminal electron acceptor was observed in growth experiments with *S. frigidimarina* NCIMB400. The results obtained are identical to those given in chapter 4: Wild type *S. frigidimarina* NCIMB400 was capable of growth on:

(i) acetate and either Fe^{3+} (as iron III citrate), NO_3^- (as KNO_3), fumarate, tetrathionate, sulphite, or glycine;
(ii) formate and either O_2, Fe^{3+} (as iron III sulphate), NO_3^-, DMSO or fumarate;
(iii) lactate and either O_2, Fe^{3+}, DMSO or fumarate;
(iv) pyruvate and either O_2, NO_2^-, TMAO, fumarate, tetrathionate, sulphite, or glycine;
(v) succinate and either O_2, NO_3^-, NO_2^-, TMAO, tetrathionate, sulphite, or glycine.

Growth characteristics of the flavocytochrome c_3 deletion strain, EG301, were identical to *S. frigidimarina* NCIMB400 with the exception of coupling between:

(i) acetate and fumarate;
(ii) formate and fumarate;
(iii) pyruvate and fumarate.

Growth characteristics of the cytochrome c_3 deletion strains, AH301 and AH401, were similar to those of NCIMB400 and EG301, respectively, with the exception of the coupling between:

(i) acetate and iron (III) citrate;
(ii) lactate and iron (III) citrate;
(iii) lactate and iron (III) sulphate.

All *S. frigidimarina* strains tested, both wild-type and deletion strains, were unable to form colonies on minimal media supplemented with each carbon source alone, each electron acceptor alone, or on unsupplemented media, under anaerobic conditions (Tables 5.1-5.5).

Minimal media plates supplemented with electron acceptors only were also incubated under a N_2 atmosphere and under a H_2/N_2 atmosphere, to ensure no contribution to growth by H_2 or CO_2. No growth was observed on any plate following 8 weeks incubation at room temperature.

5.2.2 Liquid media assays

Following analysis of the anaerobic respiratory capacity of the cytochrome c_3 deletion strains using the above plate assays, a recurring characteristic of the growth pattern was found to be the lack of ability to form colonies on plates containing acetate or formate and iron (III), where wild-type grew well. Wild-type and mutant strains were subsequently used to inoculate Luria broth medium supplemented with 15 mM formate and 50 mM iron (III) citrate. Following a 2 week anaerobic incubation period at 23 °C, an obvious growth deficiency of the mutant strains was observed; wild-type *S. frigidimarina* NCIMB400 grew well, utilising the iron in the medium, whereas the deletion strains failed to dissimilate the iron (Figure 5.5).

Figure 5.5. Phenotypic Characterisation of Cytochrome c_3: Growth of wild-type and cytochrome c_3 deletion strains of *Shewanella frigidimarina* NCIMB400 in Ferric Citrate Medium

In order to obtain quantitative data on these differences, a liquid medium assay was then used. This employed the same minimal medium and concentrations of supplements used in the plate assay. 15 ml liquid cultures in 20 ml bijoux bottles were inoculated with a 500 µl overnight LB culture of the appropriate strain. Bottles were tightly sealed and seals wrapped with Nesco film to prevent oxygen leaking into the bijoux, then incubated on a rotating platform at 23 °C for 7 days. Growth was then estimated by measuring the optical density at 500 nm of a 1ml aliquot of medium.

Table 5.6 Phenotypic characterisation of cytochrome c_3 deletion strains. Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **acetate** as the carbon source. Absorbance at 500 nm expressed as mean ± σ (standard error; n = 6). Uninoculated media used as "blank".

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	0.273 ± 0.011	0.243 ± 0.013	0.280 ± 0.01	0.257 ± 0.006
Iron (III) citrate	0.114 ± 0.005	0.055 ± 0.001	0.150 ± 0.003	0.0685 ± 0.004
Iron (III) chloride	0.97 ± 0.005	1.0215 ± 0.007	1.032 ± 0.005	0.9735 ± 0.01
Nitrite	0.116 ±0.003	0.0945 ±0.005	0.061 ±0.005	0.0795 ±0.001

Table 5.7 Phenotypic characterisation of cytochrome c_3 deletion strains. Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **formate** as the carbon source. Absorbance at 500 nm expressed as mean ± σ (standard error; n = 6). Uninoculated media used as "blank".

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	0.536 ± 0.0006	0.512 ± 0.0008	0.498 ± 0.002	0.5125 ± 0.003
Iron (III) citrate	0.098 ± 0.0004	0.0285 ± 0.0006	0.05 ± 0.001	0.0295 ± 0.0002
Iron (III) chloride	1.1 ± 0.007	1.0495 ± 0.01	1.039 ± 0.0001	1.071 ± 0.0002
Nitrite	0.106 ± 0.003	0.0895 ± 0.0004	0.09 ± 0.001	0.082 ± 0.002

Table 5.8 Phenotypic characterisation of cytochrome c_3 deletion strains. Growth of wild type *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **lactate** as the carbon source. Absorbance at 500 nm expressed as mean ± σ (standard error; n = 6). Uninoculated media used as "blank".

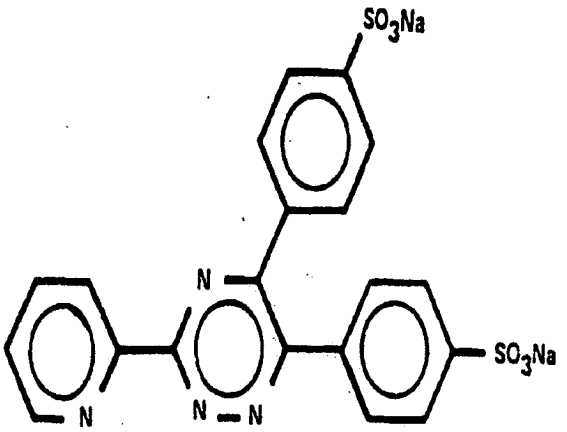
	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	0.102 ± 0.004	0.074 ± 0.06	0.098 ± 0.001	0.112 ± 0.006
Iron (III) citrate	0.13 ± 0.03	0.0425 ± 0.004	0.048 ± 0.003	0.0380 ± 0.0004
Iron (III) chloride	0.058 ± 0.004	0.0795 ± 0.007	0.032 ± 0.006	0.131 ± 0.008
Nitrite	0.056 ± 0.001	0.04 ± 0.002	0.032 ± 0.005	0.05 ± 0.007

As shown in tables 5.6-5.8, a significant decrease in total growth of both *S. frigidimarina* cytochrome c_3 deletion strains may be found using iron (III) citrate as the source of iron (III), regardless of the organic carbon source. Control experiments using two relatively insoluble iron sources, iron (III) sulphate and iron (III) chloride, showed no significant differences between wild-type and deletion strains. Similarly, no significant differences were found between strains cultured in nitrite-supplemented media; as expected from the lack of colony formation on solid media (section 5.2.1).

5.2.3 Ferrozine Assays

Results of the above characterisation experiments all point to a role for *S. frigidimarina* NCIMB400 cytochrome c_3 in respiration using iron (III) as the terminal electron acceptor. In order to investigate this further, a technique was developed to determine the reduction of iron (III) to iron (II) in anaerobic cultures of both wild-type and cytochrome c_3 deletion strains of *S. frigidimarina* NCIMB400. The assay is based on the short term extraction of iron (II) by ferrozine. Ferrozine is a bidentate ligand commonly used as a spectrophotometric reagent for iron (II) (Figure 5.6). It reacts with divalent iron to form a stable magenta complex species which is

Figure 5.6. Structure of Ferrozine. **Stookey, 1970.**



very soluble in water (Stookey, 1970). Complex formation may be monitored by following the change in absorbance at 562 nm. As with all chelators based on the ferroin moiety, the affinity of ferrozine for iron (III) is negligible.

5.2.3.1 Ferrozine assays of spent media

A 5 ml overnight culture was used to inoculate 150 ml of minimal medium with 50 mM iron (III) as iron (III) citrate, iron (III) chloride, or iron (III) sulphate, and with 15 mM acetate, formate, or lactate. The bottles were then filled to the brim with media, sealed with Nesco film and incubated at 23 °C on a rotating platform.

Following 2 weeks incubation, 2 ml aliquots of media were assessed for differences in iron (II) content as described in chapter 2. Results are given in tables 5.9 – 5.11.

Table 5.9 Phenotypic characterisation of cytochrome c_3 deletion strains. Iron (II) content of media following 2 weeks incubation of *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **acetate** as the carbon source. The reduced iron content [μg iron (II)] in each aliquot was calculated from absorbance at 562 nm using a number of iron standards (see appendix III). Data expressed as mean ± σ (standard error; n = 6). Uninoculated medium used as "blank".

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	1.90 ± 0.02	1.76 ± 0.013	1.88 ± 0.009	1.06 ± 0.05
Iron (III) citrate	4.955 ± 0.35	1.84 ± 0.03	3.24 ± 0.05	2.325 ± 0.12
Iron (III) chloride	1.79 ± 0.02	1.05 ± 0.004	5.53 ± 0.02	5.175 ± 0.06

Table 5.10 Phenotypic characterisation of cytochrome c_3 deletion strains. Iron II content of media following 2 weeks incubation of *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **formate** as the carbon source. The reduced iron content [μg iron (II)] in each aliquot was calculated from absorbance at 562 nm using a number of iron standards (see appendix III). Data expressed as mean ± σ (standard error; n = 6). Uninoculated medium used as "blank".

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	1.95 ± 0.02	1.90 ± 0.009	5.86 ± 0.06	2.74 ± 0.06
Iron (III) citrate	0.27 ± 0.01	0.22 ± 0.01	5.85 ± 0.013	2.153 ± 0.009
Iron (III) chloride	1.53 ± 0.009	1.08 ± 0.004	0.53 ± 0.02	0.092 ± 0.002

Table 5.11 Phenotypic characterisation of cytochrome c_3 deletion strains. Iron II content of media following 2 weeks incubation of *Shewanella frigidimarina* NCIMB400, AH301, EG301 and AH401 in liquid media using **lactate** as the carbon source. The reduced iron content [μg iron (II)] in each aliquot was calculated from absorbance at 562 nm using a number of iron standards (see appendix III). Data expressed as mean ± σ (standard error; n = 6). Uninoculated medium used as "blank".

	Strain			
Electron Acceptor	**NCIMB400 (wild-type)**	**AH301 (Δc_3)**	**EG301 (Δfcc_3)**	**AH401 ($\Delta fcc_3;\Delta c_3$)**
Iron (III) sulphate	1.335 ± 0.008	1.334 ± 0.008	1.31 ± 0.02	1.24 ± 0.006
Iron (III) citrate	1.32 ± 0.04	1.049 ± 0.02	1.306 ± 0.001	0.687 ± 0.08
Iron (III) chloride	1.32 ± 0.05	1.2985 ± 0.013	0.97 ± 0.04	1.165 ± 0.01

In each case, the "blank" used in determining the absorbance at 562 nm consisted of uninoculated medium incubated alongside the inoculated cultures. This is to ensure that any iron (II) produced results from bacterial dissimilatory iron (III) reduction.

As shown in tables 5.9 – 5.11, the production of iron (II) varies considerably depending on the source of iron (III). Dobbin *et al.* (1995) examined the ability of *Shewanella putrefaciens* NCIMB10471 to reduce iron (III) complexed to a variety of ligands. Their work indicates that the enzymes responsible for iron (III) reduction

lack substrate specificity. However, soluble iron (III) complexes are reduced at a faster rate than insoluble complexes. In this experiment, three different iron (III) sources were tested: iron (III) sulphate, iron (III) citrate, and iron (III) chloride. Of these, iron (III) citrate is the most soluble; iron (III) sulphate and iron (III) chloride represent two relatively insoluble sources of iron (III). It may be noted from the results in tables 5.9 – 5.11 that there are no significant differences in iron (II) production by wild-type or recombinant strains cultured in media supplemented with iron (III) sulphate, iron (III) citrate or iron (III) chloride.

Clearly, a number of discrepancies are found in iron (II) production between wild-type and cytochrome c_3 deletion strains. These results suggest that, as found with growth experiments, cytochrome c_3 is involved in respiratory iron (III) reduction in *Shewanella frigidimarina* NCIMB400. In addition, loss of cytochrome c_3, and subsequent loss in iron (III) reduction ability, cannot be compensated by the presence of flavocytochrome c_3; both AH301 and AH401, which lack cytochrome c_3 and both flavocytochrome c_3 and cytochrome c_3 respectively, consistently display similar losses in iron (II) production.

5.2.3.2 Whole cell ferrozine assays

In the above assay, the iron (II) content of media was assessed after incubation of wild-type and cytochrome c_3 deletion strains. As with cell growth experiments, some significant differences were noted between the various strains. However, in order to eliminate any possible interference of the media with the ferrozine assays above, and also to enable a more detailed examination of the iron reduction process, further experiments with whole cells from each strain are necessary.

To this end, Luria broth cultures, unsupplemented, of both wild-type and mutant strains were inoculated. Following 2 weeks anaerobic growth, cells were harvested, washed in HEPES buffer, and the pellet weighed. Approximately 0.5 g of each pellet was then resuspended in HEPES buffer for use in ferrozine assays (Chapter 2; section 2.5). Anaerobic assays on 30 – 100 µl aliquots of this cell suspension were performed in 2 ml suba-sealed cuvettes containing 50 mM NaHEPES (pH 7.0) plus 350 µM

ferrozine, initiated by the injection of 100 μM iron (III) as iron (III) citrate. Additions of formate or lactate (500 μM) were made 5 minutes before iron (III).
Following injection of iron (III), cuvettes were inverted several times to thoroughly mix the contents. The accumulation of iron (II) was then monitored over a 25 minute period by following the change in absorbance at 562 nm. Both *S. frigidimarina* NCIMB400 and EG301 strains were examined in triplicate together with their respective cytochrome c_3 deletion strains, AH301 and AH401. Results for formate and lactate are given in figures 5.7 and 5.8 respectively.

Figure 5.7. Reduction of iron (III) by *S. frigidimarina* NCIMB400, EG301(Δ*fcc*$_3$), AH301(Δc_3) and AH401(Δ*fcc*$_3$:Δc_3). Iron (II) concentration (μg), calculated from the change in absorbance at 562 nm measured from each strain in triplicate, is given following addition of 500 μM **formate** and 100 μM iron (III) citrate. Identical mixtures, replacing cell suspension with HEPES buffer, were used as "blanks".

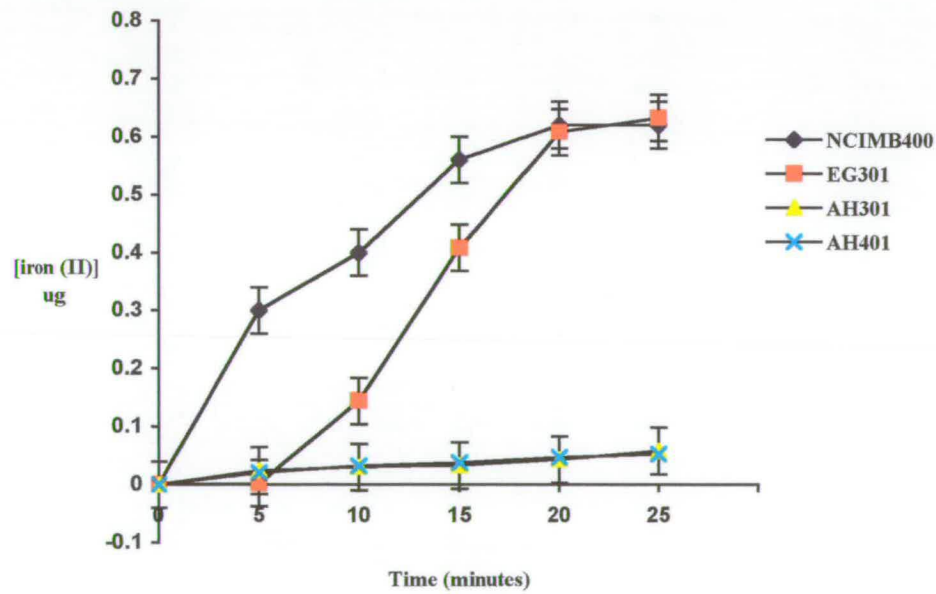
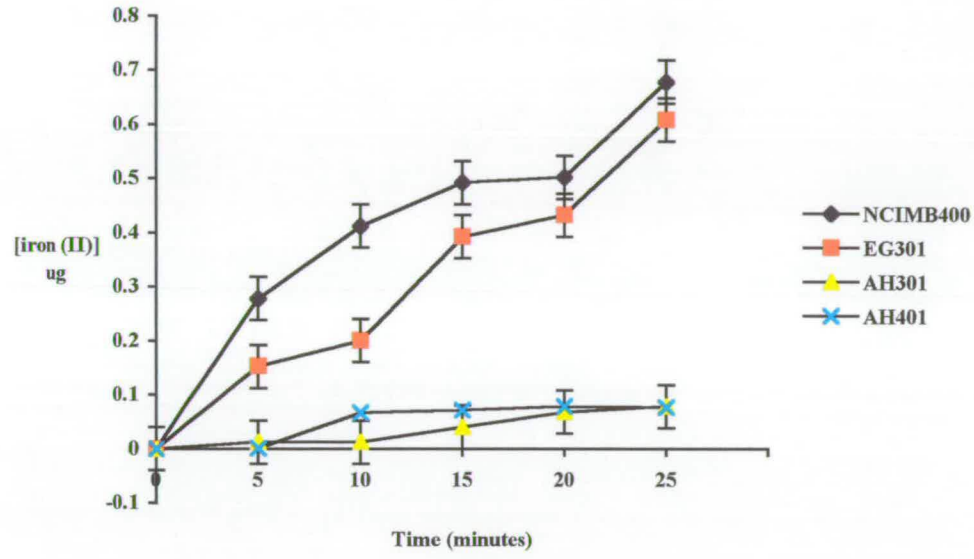


Figure 5.8. Reduction of iron (III) by *S. frigidimarina* NCIMB400, EG301(Δfcc_3), AH301(Δc_3) and AH401(Δfcc_3, Δc_3). Iron (II) concentration (μg), calculated from the change in absorbance at 562 nm measured from each strain in triplicate, is given following addition of 500 μM **lactate** and 100 μM iron (III) citrate. Identical mixtures, replacing cell suspension with HEPES buffer, were used as "blanks".



As shown in figures 5.7 and 5.8, the ability of cytochrome c_3 deletion strains to reduce iron (III) is significantly lower than the respective wild-type strains. Approximately seven-fold less iron (II) is produced by the deletion strains AH301 and AH401 over this time-period. More significantly, evidence is provided that indicates iron (III) reduction does still occur in deletion strains, albeit at a low level; for both AH301 and AH401 a steady increase in iron (II) is observed (Figure 5.7 and 5.8).

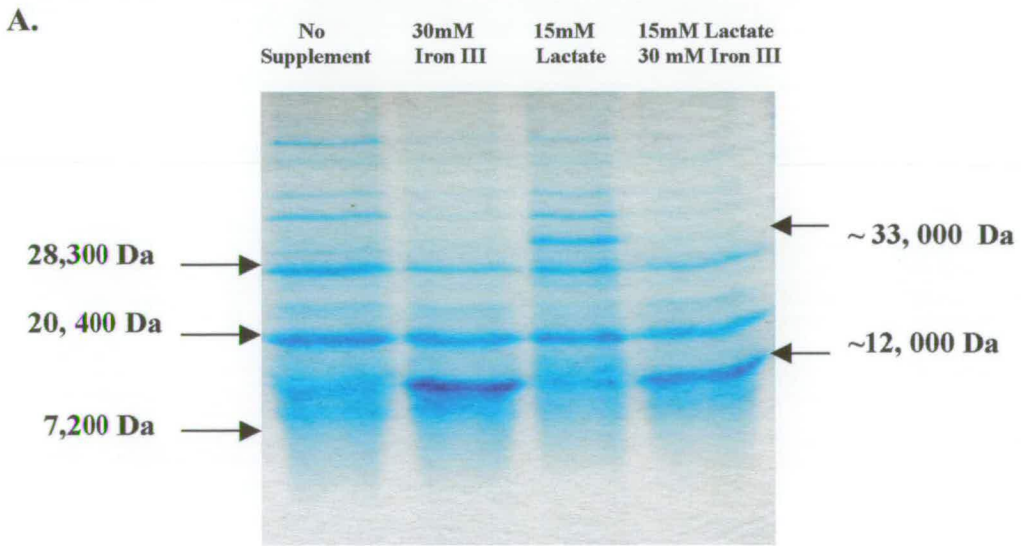
These results reinforce the suggestion that cytochrome c_3 plays a role in iron (III) reduction, but also that the pathway in which cytochrome c_3 participates is not the only pathway terminating in iron (III) reduction.

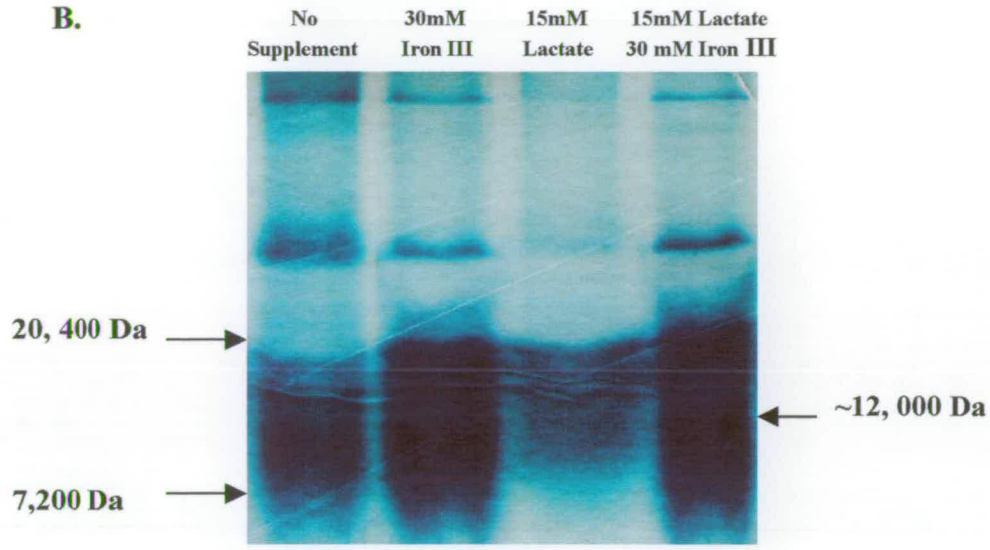
Again, flavocytochrome c_3 cannot compensate for the absence of cytochrome c_3; rates of iron (II) accumulation for EG301 and AH401 are comparable to those of NCIMB400 and AH301, respectively.

5.3 Induction of *Shewanella frigidimarina* NCIMB400 cytochrome c_3 by iron III

If *Shewanella frigidimarina* NCIMB400 cytochrome c_3 does have a role, even a non-obligatory one, in an electron transfer pathway to iron III reduction, it might be expected that its synthesis would be elevated above a basal level following growth under conditions in which these types of enzymes are themselves induced. For this reason, wild type *S. frigidimarina* NCIMB400 cells were used to inoculate Luria Bertani medium supplemented with lactate as the organic carbon source, and iron III citrate as a source of iron III. Following 2 weeks of anaerobic incubation 500 ml cultures were harvested and soluble fractions examined by SDS-PAGE and haem staining (Figure 5.9).

Figure 5.9. Influence of iron III on expression of *c*-type cytochromes in *Shewanella frigidimarina* NCIMB400. Soluble fractions were examined following two weeks growth with the appropriate supplements. Unsupplemented LB cultures are provided as controls.
A. 12% SDS-PAGE gel, stained with Coomassie brilliant blue.
B. 12% SDS-PAGE gel, stained for haem.





As shown in figure 5.9 A, a number of proteins are induced upon addition of iron (III) citrate to the growth medium. One of these proteins, of the approximate size of cytochrome c_3 (Mw = 11, 780 Da), is strongly induced under these conditions. Subsequent haem stain analysis demonstrated that this protein is a *c*-type cytochrome (Figure 5.9 B).

The addition of lactate to the growth medium did not affect protein levels. However, as shown in figure 5.9 A, an approximately 33,000 Da protein appeared to be induced upon the addition of lactate, and could therefore be involved in lactate utilisation. Alternatively the presence of Fe (III) could prevent induction of the 33 kDa protein, the reasons for this are unknown. No bands are observed of this molecular weight on haem staining; this protein is not a *c*-type cytochrome (Figure 5.9 B).

It was noticeable during this experiment that cultures supplemented with lactate and iron III citrate took several days to reach a density great enough for harvesting. Attempts to grow *S. putrefaciens* anaerobically with lactate as the electron donor and iron III citrate as the electron acceptor by Dobbin *et al.* (1995) also resulted in low biomass following 2 weeks of incubation. The reasons for this are unknown.

5.4 Discussion

Two *Shewanella frigidimarina* cytochrome c_3 deletion strains have been constructed via homologous recombination between the chromosomal *cct*A gene and a defective *cct*A gene on a suicide plasmid. The deletion strains were verified by Southern blotting and SDS PAGE.

The physiological function of cytochrome c_3 was then assessed by testing the ability of the deletion strains to grow anaerobically using a variety of electron donors and acceptors. The mutant strains share identical anaerobic respiratory capacity with respect to their corresponding c_3-wild type strains, with the exception of coupling between acetate, formate and lactate oxidation to iron (III) reduction.

The capacity of wild-type and c_3 deletion strains to reduce iron (III) to iron (II) was then determined using a variety of assays based on the extraction of ferrozine. Assessment of the iron (II) concentration of media following anaerobic growth of all strains demonstrated a reduction in iron (II) formation by both cytochrome c_3 deletion strains (AH301 and AH401) with respect to wild type following two weeks anaerobic incubation. Iron (II) formation decreased by a factor of approximately 2 in deletion strain cultures. Similarly, assays using washed, whole cells, demonstrated that cytochrome c_3 deletion strains reduced significantly less iron (III) citrate with respect to wild type over a period of 30 minutes.

Examination of soluble fractions of *Shewanella frigidimarina* NCIMB400 following growth in media supplemented with lactate and iron (III) citrate indicated that expression of a number of cytochromes is increased by the addition of these supplements. One of these cytochromes is of the size of cytochrome c_3.
It seems possible that the addition of iron to the growth medium increases cytochrome formation in general due to the necessity of iron in cytochromes. However, work by Morris (1987) identified no fewer than nine *c*-type cytochromes in *S. frigidimarina* NCIMB400. Only three *c*-type cytochromes were visibly induced in supplemented cultures.

The use of iron (III) as an electron acceptor by microorganisms has important environmental implications. For example, biogeochemical cycling of iron is thought to play a significant role in the aquatic distribution of other elements such as carbon, nitrogen, sulphur, and phosphorus. As a result, microbial iron (III) reduction has been intensively studied over recent years (DiChristina and DeLong, 1994; Lovely, 1991; Lovely, 1993; Lovely, 1995; Slobodkin *et al.*, 1997; Myers and Myers, 1993). A number of genera of specifically iron-reducing bacteria have been identified. These include *Geobacter*, *Desulfuromonas*, *Bacillus*, *Ferrimonas*, and *Shewanella* (Seeliger *et al.*, 1998; Slobodkin *et al.*, 1997). Studies with defined cultures of these bacteria have shown that they can specifically couple iron reduction to the oxidation of a broad range of substrates.

For *Shewanella*, both biochemical and genetic techniques have been employed to examine the components of the electron transport chain to iron (III). Mutants with lowered iron (III) reductase activity have been found to possess suppressed cytochrome levels (DiChristina *et al.*, 1988). In addition, elevation of *b*-, *c*-, and *d*-type cytochromes in anaerobic or microaerobic cultures of *Shewanella putrefaciens* are accompanied by a marked increase in iron (III) reductase activity in comparison with fully aerobic cultures (Dobbin *et al.*, 1995; Myers and Myers, 1992; Obuekwe and Westlake, 1982). Sensitivity of iron (III) reduction rates to 2-heptyl-4-hydroxyquinolone-N-oxide (HQNO) also suggests the involvement of a quinol: cytochrome oxidoreductase (Myers and Myers, 1993). Finally, a tetrahaem cytochrome *c* required for reduction of iron (III), fumarate, and nitrate has recently been identified in *S. putrefaciens* MR-1 (Myers and Myers, 1997).

Tsapin *et al.* (1998) have isolated a small, tetrahaem cytochrome *c* from the periplasm of *Shewanella putrefaciens* MR-1. This protein shares a number of features with cytochrome c_3 isolated from *S. frigidimarina* NCIMB400, including a close sequence similarity, and an unusually low redox potential. The function of the protein from MR-1 has not been investigated, however, the reduced form of this cytochrome was rapidly oxidised by iron (III) citrate.

Recently a periplasmic and extracellular *c*-type cytochrome from *Geobacter sulfurreducens* has been described by Seeliger *et al.* (1998). The proposed function of this cytochrome is as an iron (III) reductase, that is capable of transferring electrons to other acceptor systems. This is a low molecular weight tri-haem cytochrome *c* similar to the cytochromes *c* from sulphate reducing bacteria. It has a comparable midpoint potential of –167 mV, and reacts unspecifically with a broad variety of electron acceptors including oxygen, iron (III) citrate, iron (III) nitrilotriacetic acid, manganese oxide, and sulfur. Seeliger *et al.* (1998) propose that this cytochrome acts as a redox partner to humic acids and partner bacteria. The idea that this cytochrome is excreted seems rather wasteful as it seems difficult to imagine that an efficient electron transport chain could be set up.

Historically, cytochromes c_3 have been implicated in sulphate respiration, due to the isolation of these proteins exclusively from sulphate reducing bacteria. Cytochromes c_3 are thought to be involved in the redox transfer of hydrogen to the cytoplasmic reduction of sulphate (Aubert *et al.*, 1997; Czjzek *et al.*, 1996; Pieulle *et al.*, 1996; Saraiva *et al.*, 1996). If we examine the respiratory capacity of *Shewanella*, however, we find that they do not couple growth to the reduction of sulphate. Why then, is cytochrome c_3 produced by these organisms? It seems possible that cytochrome c_3 may act as a charge separation device in *Shewanella*, similar to that described for *Desulfovibrio* (see section 1.5.3.1), this time donating electrons to an electron transport chain terminating with iron (III), as opposed to sulphate.

If we consider redox potential, the value of approximately – 230 mV, given for both of these *Shewanella* cytochromes c_3, should allow them to reduce almost any terminal electron acceptor. It does not seem surprising therefore that iron (III) citrate acts as an efficient electron acceptor in *in vitro* experiments with purified cytochrome c_3. However, the significant decrease in iron (III) reduction found using cytochrome c_3 deletion strains, described in this chapter, indicate that in *Shewanella frigidimarina* NCIMB400, cytochrome c_3 does have a role in coupling growth to iron (III) reduction.

In a number of studies no differences in the phenotype of mutant strains in comparison to wild-type have been found. The conclusion derived from such studies has been that another protein is substituting for the knocked-out protein. Owing to the similarity between cytochrome c_3 and the cytochrome domain of flavocytochrome c_3 from *Shewanella frigidimarina* NCIMB400, the effect of deleting both of these cytochromes was investigated. This was to determine whether flavocytochrome c_3 in any way substitutes for cytochrome c_3. As shown in both growth experiments and iron (III) reduction assays, few differences may be found between the single and double-deletion strains. This indicates that flavocytochrome c_3 cannot substitute for cytochrome c_3 and also that flavocytochrome c_3 does not act in any other pathway to iron (III) reduction.

If we consider the make-up of bacterial electron transport chains, the basic modular arrangement of a respiratory pathway consists of a substrate oxidase, a quinone, and a terminal reductase. The midpoint potentials of these components are poised such that each member can be reduced by the reduced form of the preceding member. The midpoint of menaquinone is – 74 mV. This value is much greater than the midpoint of cytochrome c_3, which would not be reduced by this quinone. A number of quinone derivatives have been isolated from *Shewanella* (Tsapin *et al.*, 1996). It will be interesting to find out whether the midpoint potentials of these respiratory components are in a range more suitable for involvement of cytochrome c_3.

Saffarini and Nealson (1993) indicated that the ability of *Shewanella putrefaciens* to grow on iron (III) is governed by the gene *etr*A, the *fnr* homologue. However, *etr*A mutants retain the ability to reduce iron (III) at rates comparable to the wild-type, reinforcing the hypothesis that more than one iron (III) reductase may exist. In line with this, no putative *etr*A site was identified upstream of the cytochrome c_3 structural gene.

Myers and Myers (1993) have investigated iron (III) reductase activity in *Shewanella putrefaciens* MR-1, and localised all activity to the outer membrane. Further to this, Dobbin *et al.* (1996), during investigation of the effects of iron (III) speciation on

dissimilatory iron reduction, have localised iron reductase activity to both the outer membrane and the cytoplasmic membrane, confirming the suspicion that *Shewanella putrefaciens* MR-1 contains more than one iron (III) reductase. Indeed, the results described in this chapter are consistent with the presence of more than one terminal reductase; iron (III) reductase activity was not completely abolished by deleting cytochrome c_3.

Further examination of the respiratory pathways to iron within *Shewanella* will be greatly facilitated by the use of multiple cytochrome deletion strains. At this point, it seems reasonable to suggest that cytochrome c_3 from *Shewanella frigidimarina* NCIMB400 is involved in one or more respiratory chains linking organic substrate oxidation to iron (III) reduction.

Final Discussion and Future Work

Bacteria exhibit considerable diversity in the complexity of their respiratory chains. This diversity reflects several different properties including not only the genetic information inherent within the organism, but also the redox potentials of the donor and acceptor couples, the ability of the organism to regulate respiratory chain components, and also the extent to which the respiratory chain has to interact simultaneously or sequentially with more than one donor and/or acceptor. This diversity also serves to complicate the elucidation of the respiratory pathways of a number of organisms.

In this work a genetic approach was adopted to determine the physiological redox partners for two small cytochromes, cytochrome c_5 and cytochrome c_3, both respiratory chain components of *Shewanella frigidimarina* NCIMB400. Construction of mutant strains in which the structural genes are disrupted enabled the phenotypic effect of a lack of these cytochromes to be examined.

For cytochrome c_3 two mutant strains, AH301 and AH401, which lacked cytochrome c_3 and both cytochrome c_3 and flavocytochrome c_3 respectively, were constructed. Growth experiments showed that neither strain could consistently grow anaerobically with iron (III) as the sole terminal electron acceptor. A decrease in iron (III) reduction was also demonstrated through ferrozine extraction assays. These experiments suggest not only that cytochrome c_3 is involved in respiratory iron (III) reduction, but also that flavocytochrome c_3 cannot complement cytochrome c_3 deficiency. In addition, ferrozine extraction assays imply that more than one pathway exists linking substrate oxidation to iron (III) reduction; not all iron (III) reduction activity was abolished in the absence of cytochrome c_3. The results strengthen the importance of iron (III) reduction to this obligately respiratory organism.

Four mutant strains were constructed to examine the role of cytochrome c_5. Strain AH501 lacked cytochrome c_5 whereas strains AH601, AH701, and AH801 lacked cytochrome c_5 and flavocytochrome c_3, cytochromes c_5 and c_3, or cytochromes c_5, c_3 and flavocytochrome c_3, respectively. None of these strains was able to grow anaerobically

with nitrate as the sole terminal electron acceptor with formate as the organic carbon source. It seems reasonable to suggest that cytochrome c_5 is involved in a respiratory chain linking growth to nitrate reduction, but it may also be possible that cytochrome c_5 is involved in respiratory protection of nitrate reductase. Growth experiments with mutant strains AH501- AH801 demonstrate that cytochromes c_5, c_3 and flavocytochrome c_3 do not participate in the same respiratory chain; no additional mutant phenotypes were observed.

In order to confirm that the phenotypic effects described above are due to the disruption of *scy*A and *cct*A, and thus to the loss of cytochrome c_5 and c_3 respectively, complementation may be used; if the respiratory defects are restored by supplying the wild-type gene it can be assumed that the correct physiological role has been identified.

Further examination of the regulation of these cytochromes, through the use of reporter fusions, will undoubtedly aid the identification of the physiological role(s) of these proteins. Reporter fusions have previously been successful in the characterisation of flavocytochrome c_3 as the sole fumarate reductase in *Shewanella frigidimarina* NCIMB400 (Gordon, 1998).

One of the interesting questions raised during examination of cytochromes c_5 is the presence of an "extra" pair of cysteine residues which do not bind haem. The construction of AH501 cells will aid the investigation of the role of this cysteine pair; recombinant forms of cytochrome c_5 may be overexpressed without contamination by wild-type protein. In addition, a successful overexpression system for cytochrome c_5, using the broad-host-range vector pMMB503, has been developed.

The cytochrome c_5 overexpression system is also useful for the production of large amounts of protein for crystallisation studies. A crystal structure will allow examination of intramolecular electron transfer and, in the future, a look at the interaction between physiological redox partners.

The immediate concern in the investigation of the respiratory pathways in *Shewanella* is the production of multiple respiratory chain component knock-out strains. In particular, for the investigation of pathways involving cytochrome c_5, a cytochrome c_4 deletion strain is necessary. Both cytochrome c_4 and cytochrome c_5 are small, high potential, periplasmic proteins. It seems important to rule out any possible functional overlap between these two cytochromes. The production of deletion strains, and the examination of respiratory chains components, will be greatly facilitated by the imminent publication of the complete *Shewanella putrefaciens* MR-1 genome sequence.

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Genetic markers

Genetic Marker	Description
ara	Inability to utilise arabinose
*arg*F	Inability to utilise arginine; ornithine carbamoyltransferase mutation
dam	Blocks adenine methylation at GATC sequences; DNA adenine methylase mutation
dcm	Blocks cytosine methylation at CCAGG or CCTGG sequences:DNA cytosine methylase mutation
*deo*R	Allows uptake of large plasmids; regulatory gene mutation allowing constitutive expression of genes for deoxyribose synthesis
*end*A	Abolishes nonspecific endonuclease I activity; improves quality of plasmid DNA isolations
*gal*K	Inability to utilise galactose; galactokinase mutation
*gal*T	Inability to utilise galactose; galactose-1-phosphate uridylyltransferase mutation
*gal*U	Inability to utilise galactose; glucose-1-phosphate uridylyltransferase mutation
*gyr*A	Resistance to nalidixic acid; DNA gyrase mutation
*hfl*A	High frequency lysogenisation by λ
*hsd*R	Restriction minus, modification positive; transformed DNA will not be cleaved by endogenous restriction endonucleases
*hsd*S	Restriction minus, modification positive; transformed DNA will not be cleaved by endogenous restriction endonucleases; protective methylation abolished
*lac*I^q	Overproduction of *lac* repressor protein, leading to inhibition of transcription from the *lac* promotor
*lac*Y	Inability to use lactose; galactosidase permease mutation
*lac*ZΔM15	Allows α-complementation of β-galactosidase activity; partial deletion of β-D-galactosidase
*leu*B	Requires leucine for growth on minimal media; β-isopropyl malate dehydrogenase mutation
*mcr*A	Blocks restriction of methyl-cytosine-specific DNA sequences
*mcr*B	Blocks restriction of methyl-cytosine-specific DNA sequences
*met*B	Requires methionine for growth on minimal media; cystathionine γ–synthase mutation
mrr	Blocks restriction of methyl-adenine-specific DNA sequences
mtl	Inability to utilise mannitol
*pro*AB	Requires proline for growth on minimal media
*rec*A	Prevents recombination between introduced DNA and host DNA; confers UV light sensitivity
*rel*A	Allows RNA synthesis in the absence of protein synthesis
*rps*L	Resistance to streptomycin; mutation in protein S12 of 30S ribosomal subunit
*sbc*BC	Allows general recombination in *rec*BC mutant hosts; exonuclease I mutation
*sup*E	Suppresses amber (UAG) mutations
*sup*F	Suppresses amber (UAG) mutations; required for growth of some phage vectors
thi-1	Requires thiamine for growth on minimal media
thr	Requires threonine for growth on minimal media
*Tn*5	Transposon which encodes kanamycin resistance
*Tn*10	Transposon which encodes tetracycline resistance
*ton*A	Resistance to bacteriophage T1; mutation in outer membrane protein
*tra*D	Prevents transfer of F' episome
*trp*R	Requires tryptophan for growth on minimal media
tsx	Resistance to phage T6 and colicin K
xyl-5	Inability to utilise xylose
Δlon	Increases fusion protein stability; mutation in *lon* protease

Shewanella codon preferences

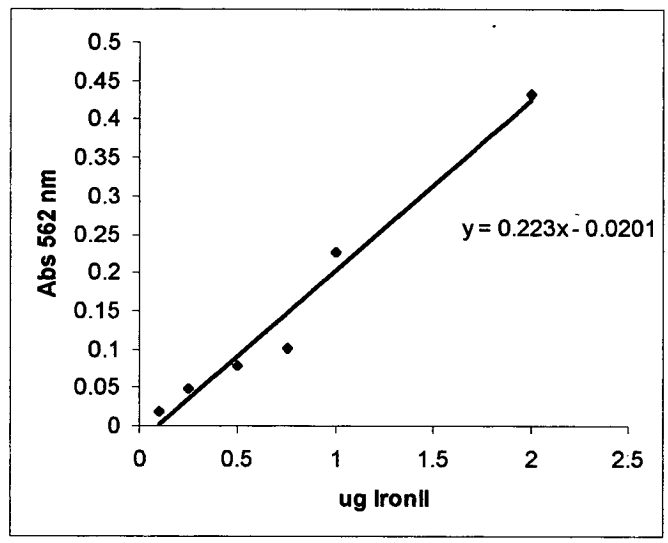
Amino acid	Codon	Number	/1000	Fraction
Gly	GGG	7.00	3.25	0.04
Gly	GGA	13.00	6.03	0.07
Gly	GGT	115.00	53.36	0.61
Gly	GGC	52.00	24.13	0.28
Glu	GAG	22.00	10.21	0.21
Glu	GAA	84.00	38.98	0.79
Asp	GAT	76.00	35.27	0.54
Asp	GAC	65.00	30.16	0.46
Val	GTG	44.00	20.42	0.27
Val	GTA	41.00	19.03	0.25
Val	GTT	59.00	27.38	0.37
Val	GTC	17.00	7.89	0.11
Ala	GCG	41.00	19.03	0.20
Ala	GCA	56.00	25.99	0.28
Ala	GCT	84.00	38.98	0.42
Ala	GCC	21.00	9.74	0.10
Arg	AGG	2.00	0.93	0.02
Arg	AGA	1.00	0.46	0.01
Ser	AGT	19.00	8.82	0.14
Ser	AGC	26.00	12.06	0.19
Lys	AAG	37.00	17.17	0.31
Lys	AAA	84.00	38.98	0.69
Asn	AAT	39.00	18.10	0.46
Asn	AAC	46.00	21.35	0.54
Met	ATG	66.00	30.63	1.00
Ile	ATA	10.00	4.64	0.09
Ile	ATT	66.00	30.63	0.57
Ile	ATC	40.00	18.56	0.34
Thr	ACG	11.00	5.10	0.09
Thr	ACA	23.00	10.67	0.19
Thr	ACT	55.00	25.52	0.47
Thr	ACC	29.00	13.46	0.25
Trp	TGG	30.00	13.92	1.00
End	TGA	1.00	0.46	0.07
Cys	TGT	29.00	13.46	0.78
Cys	TGC	8.00	3.71	0.22
End	TAG	8.00	3.71	0.53
End	TAA	6.00	2.78	0.40
Tyr	TAT	4700	21.81	0.64
Tyr	TAC	27.00	12.53	0.36

Leu	TTG	28.00	12.99	0.14
Leu	TTA	89.00	41.30	0.45
Phe	TTT	46.00	21.35	0.57
Phe	TTC	34.00	15.78	0.43
Ser	TCG	11.00	5.10	0.08
Ser	TCA	42.00	19.49	0.31
Ser	TCT	31.00	14.39	0.23
Ser	TCC	6.00	2.78	0.04
Arg	CGG	2.00	0.93	0.02
Arg	CGA	4.00	1.86	0.04
Arg	CGT	64.00	29.70	0.72
Arg	CGC	16.00	7.42	0.18
Gln	CAG	23.00	10.67	0.34
Gln	CAA	45.00	20.88	0.66
His	CAT	35.00	16.24	0.64
His	CAC	20.00	9.28	0.36
Leu	CTG	20.00	9.28	0.10
Leu	CTA	24.00	11.14	0.12
Leu	CTT	28.00	12.99	0.14
Leu	CTC	8.00	3.71	0.04
Pro	CCG	9.00	4.18	0.12
Pro	CCA	29.00	13.46	0.40
Pro	CCT	33.00	15.31	0.46
Pro	CCC	1.00	0.46	0.01

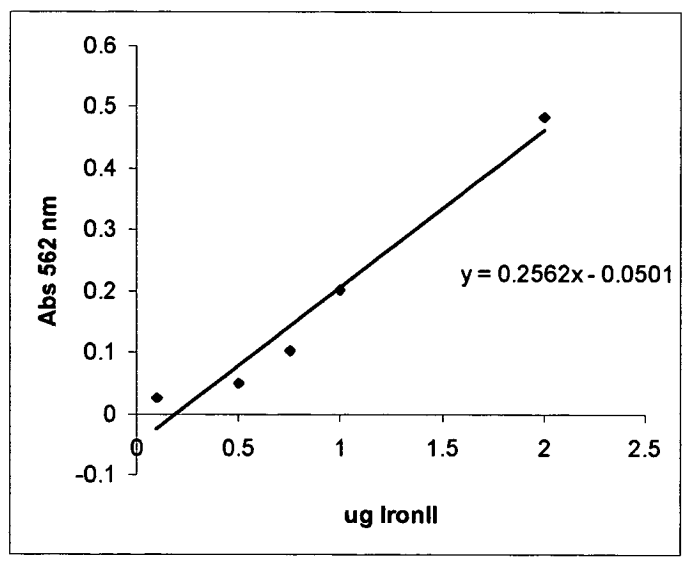
Iron (II) calibration plots

In order to determine the relative iron (II) concentrations following ferrozine extraction assays (Chapter 5), an iron (II) standard solution was used. Iron standards were made by serial dilution of 1000 µg/dL iron standard (SIGMA), in 5 % w/v hydroxylamine.HCl. The absorbance at 562 nm of each standard was measured, alongside the appropriate experiment, and used to plot the following charts:

A. Standard chart used for calculations given in tables 5.9 – 5.11 (Pages 151 and 152).



B. Standard chart used for calculations given in figure 5.7 and 5.8 (Pages154 and 155).



Details of each experiment are given in the text (Chapter 5).