FLAVOCYTOCHROME c FROM *Shewanella putrefaciens*

by

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DEDICATION

I would like to dedicate this thesis to my parents. Without their neverending support, encouragement and love, things would have been much more difficult.

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ABSTRACT

Flavocytochrome c, a multihaem cytochrome from *Shewanella putrefaciens* induced under anaerobic conditions, was studied to investigate the physiological function of this protein. These studies comprised two facets: the cloning and sequence analysis of the structural gene for flavocytochrome c and a biochemical study of the fumarate reductase activity associated with flavocytochrome c.

A *S. putrefaciens* genomic library was constructed from 0.5-4 kb *Sau*3A fragments in the expression vector pEX3. The ligated recombinant/vector library was transformed into *E. coli* MM294 to give a representative library of approximately 22,000 recombinants. This library was screened by colony hydridization of induced recombinants with antibody raised to purified flavocytochrome c protein.

One clone giving a strong signal to antibody was identified from this library. Restriction digests of the pEX3 recombinant vector purified from this clone showed the insert DNA to be approximately 1.5 kb. Southern blot analysis of the clone gave hydridization of flavocytochrome c antibody to a protein of approximately 45 kDa which was encoded by the recombinant pEX3 vector. This cloned fragment was proposed to encode part of the flavocytochrome c gene and investigated in more detail.

The 1.5 kb cloned fragment was partially sequenced and mapped. Sequencing yielded two non-overlapping contigs of 438 bp and 966 bp respectively. The DNA fragment joining the two contigs remained unsequenced. Database analysis of the predicted amino acid sequence of both contigs showed that the second contig contained 4 conserved c-type haem binding site motifs CXYCH within the first 90 residues. A second region in this contig from bases 123-151 was found to be identical with a highly conserved FAD-binding fingerprint common to many flavoproteins. Non-covalent binding of

flavin was evident by lack of an essential histidine residue directly following the conserved region. This molecular analysis strongly suggested that the cloned DNA fragment encoded at least 322 residues of the N-terminal region of the flavocytochrome c protein. This was further confirmed by the finding that the first 8 residues of the second contig were completely homologous with residues 6-13 of the N-terminal sequence of flavocytochrome c.

Cloning the entire flavocytochrome c gene was attempted also by functional complementation of an *E. coli* fumarate reductase mutation. A second chromosomal library of 4-10 kb *Sau3A* size fractionated DNA fragments was constructed in the direct selection vector pUN121. This library was transformed into and screened in JRG780, an *E. coli* mutant unable to utilise fumarate as a terminal electron acceptor under anaerobic conditions. However, complementation of fumarate reductase activity was unsuccessful.

Flavocytochrome c protein was purified to homogeneity by column electrophoresis from microaerobically grown culture on DEAE Sepharose, Hydroxyapatite, Phenyl Sepharose, and Sephacryl S-300. This purification yielded 4 mg of purified flavocytochrome c per 10 litres of culture, but was not altogether satisfactory, being rather lengthy and giving an unacceptable level of protein proteolysis. A second purification was developed which resolved these problems. It utilised precipitation of periplasm by ammonium sulphate followed by chromatography on DEAE Sepharose and Hydroxyapatite, giving a higher yield of purified flavocytochrome c (5.96 mg/10 litres of culture) over fewer stages and reducing proteolysis to a minimum.

Sheep antibody was raised to purified flavocytochrome c protein. This antibody had a relatively high affinity for flavocytochrome c, being able to detect as little as 1 ng of purified protein. This antibody was used to study fumarate reductase activity in *S. putrefaciens*: on incubation with antibody, fumarate reductase activity was almost completely precipitated from both purified flavocytochrome c and periplasm, suggesting flavocytochrome c is a single

periplasmic fumarate reductase. This was confirmed by zymogram staining of periplasm which gave a single zone of fumarate reductase activity corresponding to flavocytochrome c.

Purified flavocytochrome c had K_m and V_{max} values for fumarate of 12.6 μM and 278 μmol/min/mg protein respectively. The enzyme had a much lower affinity for succinate, with K_m being 356 μM and V_{max} 0.08 μmol/min/mg protein. This bi-directional capacity, and high specificity for fumarate is very similar to fumarate reductases in other Gram-negative bacteria and suggests a role for flavocytochrome c as a terminal fumarate reductase.

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CHAPTER 1

INTRODUCTION

THE HISTORY AND MICROBIOLOGY OF *Shewanella*

The microbial flora of marine fish caught in temperate water consists primarily of Gram-negative bacteria. During refrigeration the flora alters and bacteria belonging to the genera *Pseudomonas, Psychrobacter, Vibrio, Flavobacterium* and particularly *Shewanella* predominate. The species *Shewanella* is particularly important as it has been shown to cause the characteristics of fish spoilage in model systems (Herbert *et al*, 1970). By synthesis of TMAO reductase and its related components on oxygen depletion, growth is sustained via anaerobic respiration at the expense of TMAO which is available in marine tissues. Thus *Shewanella* may have a competitive advantage over other non-TMAO reducing bacteria during fish spoilage and assert its dominance in the spoilage flora. Although the psychrophilic *Shewanella* has been isolated from a variety of proteinaceous foods (Parker and Levin, 1983) it is primarily associated with the marine environment.

The taxonomic position of *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens*) has been uncertain for a number of years. The genus *Alteromonas* comprising heterotrophic non-fermentative bacteria was once classified with the pseudomonads (Lee *et al*, 1977). However, although biochemically similar to pseudomonads, alteromonads had to be placed in a separate genus because the base composition of their DNA (mol % GC 43-54) differs markedly from pseudomonads (mol % GC 58-72) (Baumann *et al*, 1972). Furthermore, strains of *A. putrefaciens* isolated by Gillespie (1981) showed less diversity than *Pseudomonas* spp and were characterised by their production of sulphydryl type odours, inability to produce H_2S during growth in peptone iron agar, and their ability to reduce trimethylamine oxide. Most of the alteromonads were able to utilise maltose and sucrose but not lactose or mannitol.

Van Landschoot and DeLey (1983) classified Alteromonas species into four RNA groups: (1) *Alteromonas macleodii*, (2) *Alteromonas haloplanktis*, (3) *Alteromonas putrefaciens*, (4) *Marinomonas* (*A. communis* and *A. vaga*). MacDonell and Colwel (1985) sequenced the 5s rRNA from *A. putrefaciens* ATCC 8071 (NCIB 10471) and found, that as with *A. macleodii* and *A. haloplanktis, A. putrefaciens* belonged in the first rRNA superfamily as defined by DeLey (1978), but recommended that it should be placed in a separate genus - *Shewanella*. *A. vaga* and *A. communis* were distinctly different from the other alteromonads, being part of the second rRNA and placed in a separate genus *Marinomonas*.

This consequently placed the genus *Shewanella* as more closely related to the Vibrionacea than the Enterobacteriaceae but in the same major group, rRNA superfamily 1. This agrees with Woese *et al* (1985) who placed *A. putrefaciens* DSM 20456 between the Vibrionaceae and the Enterobacteriaceae in the gamma subdivision of the purple bacteria (approximately equivalent to the rRNA superfamily 1 of Van Landschoot and DeLey).

REFERENCES

Baumann, L., Baumann, P., Mandel, M. and Allen, R.D. (1972).
Taxonomy of aerobic marine eubacteria.
Journal of Bacteriology **110**, 402-429.

DeLey, J. (1978).
Modern molecular methods in bacterial taxonomy: evaluation, application, prospects.
Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Angers, pp347-357.

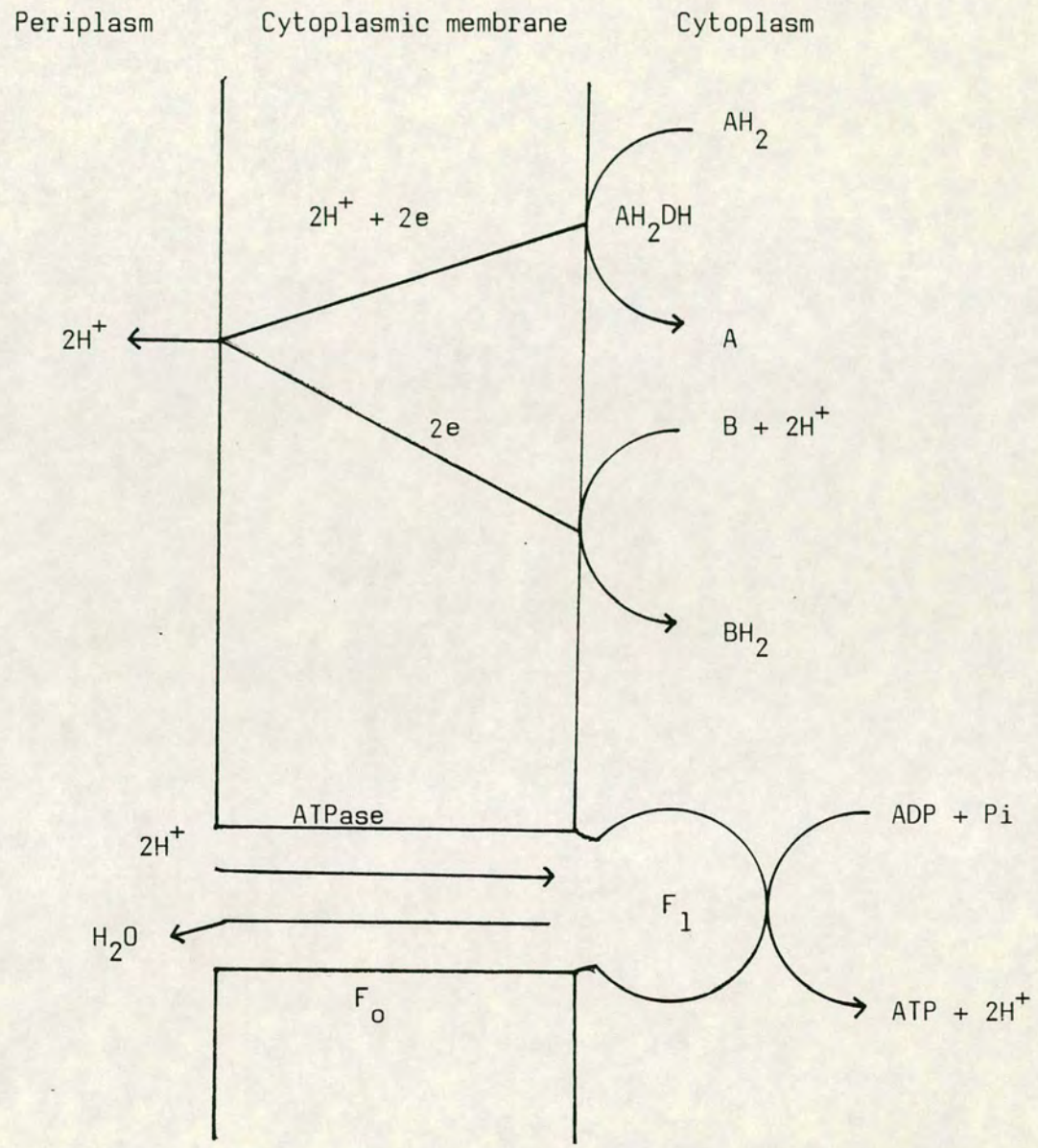
Bacteria are noted for the diversity of their habitats. The ability to survive in such a range of habitats is due to their adaptive ability on exposure to adverse external conditions, and their metabolic diversity. Part of this diversity is the ability to generate energy by a variety of mechanisms: aerobic and anaerobic respiration, fermentation and photosynthesis. The following review will focus on the diversity and control of anaerobic respiration in Gram negative bacteria, after a brief discussion of aerobic respiration and fermentation. Energy production by means other than anaerobic respiration has been extensively reviewed in the following literature: Drews, 1985; Scolnik and Marrs, 1987; Anraku and Gennis, 1987; Spencer and Guest, 1987.

1.1 Chemiosmotic theory

Energy generation via aerobic, anaerobic and photosynthetic pathways have certain common features, described in the chemiosmotic theory proposed by Mitchell (1979). In simple terms this involves an H^+-ATPase proton-pumping system, associated with an intact membrane. The transportation of protons from cytoplasm to periplasm (by a series of oxidation/reduction reactions mediated through the membrane-located electron transporting components: flavoproteins, quinones, iron-sulphur proteins and cytochromes) creates a proton-motive force (δp) with gradients of both electrical potential ($\delta\varphi$) and pH (δpH) generated across the membrane. The net result of this is the driving of protons back into the cell through a membrane bound ATPase complex, generating ATP. This process is summarised in simple diagrammatic terms in Figure 1.1.

A proton gradient can equally be created either by direct translocation or via proton consuming and proton generating reactions on the cytoplasmic and periplasmic face of the cytoplasmic membrane respectively. Recent work indicates that this theory can be extended to the generation of non-protonic ion gradients, by sodium ions (Dimroth, 1987; Krulworth and Guffanti, 1989).

Figure 1.1 Diagrammatic representation of the chemiosmotic theory



On substrate reduction, $2H^+$ are released into the periplasm, and $2e^-$ passed to a hydrogen acceptor via hydrogen and electron carriers spanning the membrane. This generates gradients of electrical potential (φ) and pH (pH) across the membrane, establishing a proton motive force (δp) which drives the generation of ATP. This diagram is simplistic: a complete electron transport chain will usually involve more than one proton translocating component (see Figure 1.5).

AH_2: substrate; AH_2DH: substrate dehydrogenase; B: hydrogen acceptor

1.2 Aerobic respiratory chain of E. coli

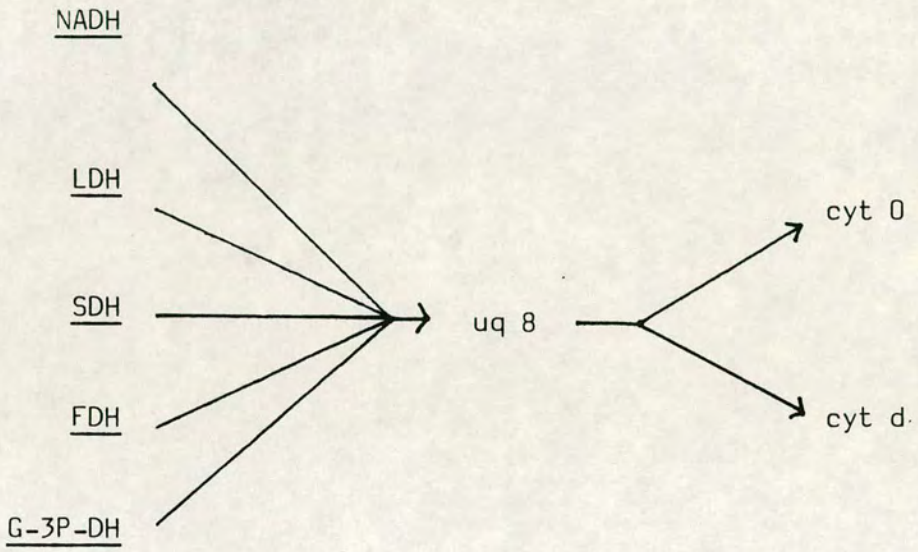
During aerobic respiration substrates are totally oxidised to CO_2 via the TCA cycle, with ATP generated by proton and electron transfer (by oxidation of reduced coenzymes) through an aerobic respiratory chain, the terminal electron acceptor being molecular oxygen. Respiratory chains of enteric bacteria can be very diverse, allowing electron transfer from one of several substrates to support respiratory chain activity. This diversity is well illustrated in the example from *E. coli* (Figure 1.2), where several substrates are able to donate electrons via membrane bound dehydrogenases to a common quinone pool. In turn, electrons are donated from the quinone pool to either of two terminal oxidase enzymes. Cytochrome o appears to be utilised preferably in conditions of high oxygen tension (Ingledew and Poole, 1984; Kita *et al*, 1984), whereas cytochrome d predominates in oxygen limited cells (Kranz and Gennis, 1984). The cytochrome composition of aerobic respiratory chains in *S. typhimurium* and *P. mirabilis* are similar to that of *E. coli* (Laszlow *et al*, 1984; van Wielink *et al*, 1983).

1.3 Effect of anaerobiosis on TCA cycle enzymes

As a consequence of oxygen limitation, the activities of the TCA cycle enzymes citrate synthase, aconitase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are drastically reduced (Smith and Neidhardt, 1983; Spencer and Guest, 1985) preventing generation of α-ketoglutarate and succinate (for biosynthesis), although succinate may be synthesised by reduction of fumarate via the reductive branch of the TCA cycle which becomes active anaerobically (Ingledew and Poole, 1984) (Figure 1.3).

In the absence of oxygen, bacteria can generate energy by two alternative strategies: (a) fermentation; or (b) anaerobic respiration.

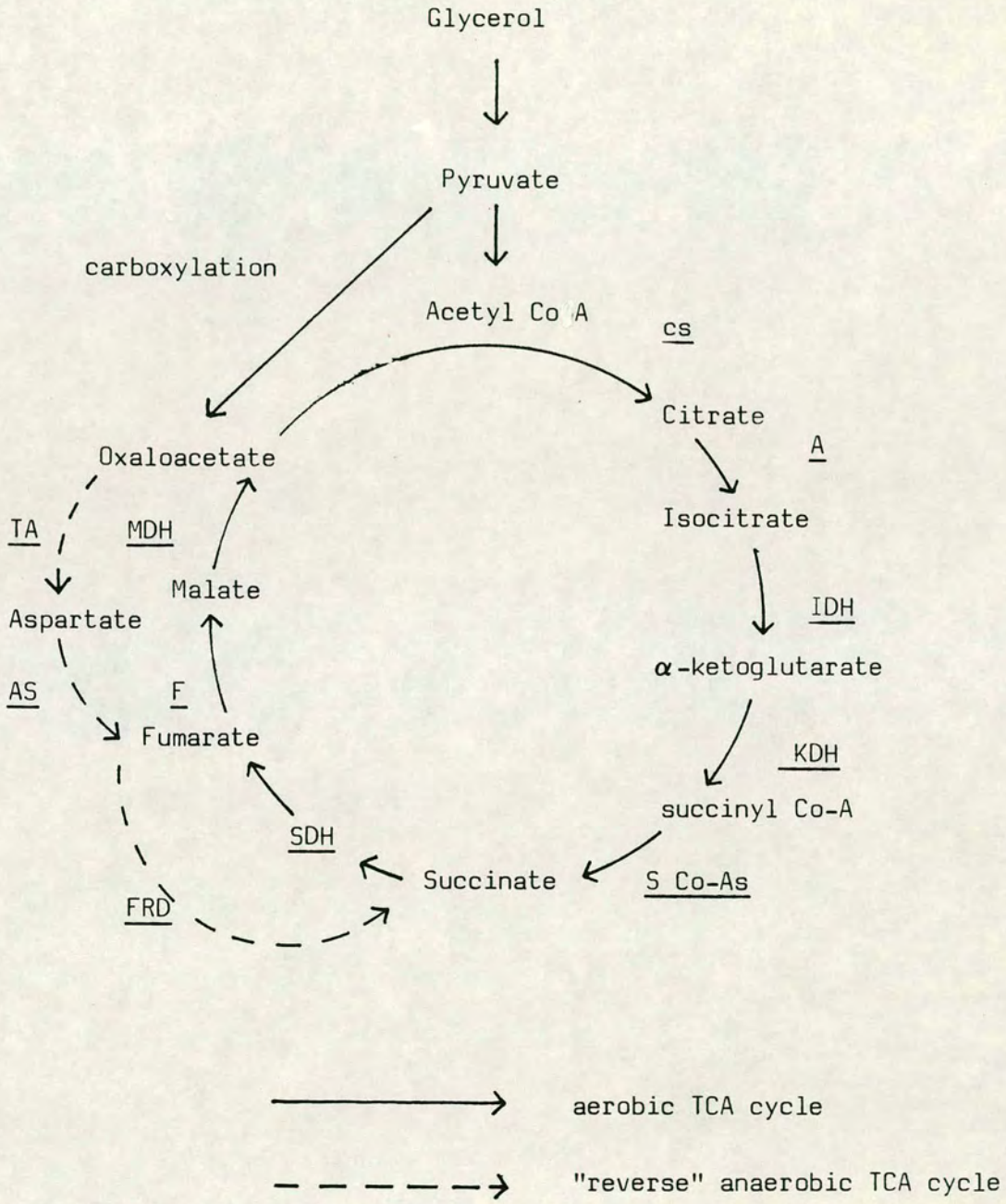
Figure 1.2 Diversity of enteric aerobic respiratory chain



Aerobic respiratory chains are branched on both sides of quinone allowing greater flexibility for oxidant and reductant use. See text for details.

NADH: nicotinamide adenine dinucleotide dehydrogenase; LDH: lactate dehydrogenase; SDH: succinate dehydrogenase; FDH: formate dehydrogenase; G-3P-DH: glycerol-3-phosphate dehydrogenase; uq: ubiquinone pool.

Figure 1.3 TCA cycle



CS: citrate synthase; A: aconitase; IDH: isocitrate dehydrogenase; αKDH: α-ketoglutarate dehydrogenase; SCo-AS: succinyl Co-A synthetase; SDH: succinate dehydrogenase; F: fumarase; MDH: malate dehydrogenase; TA: transaminase; AS: aspartase; FRD: fumarate reductase.

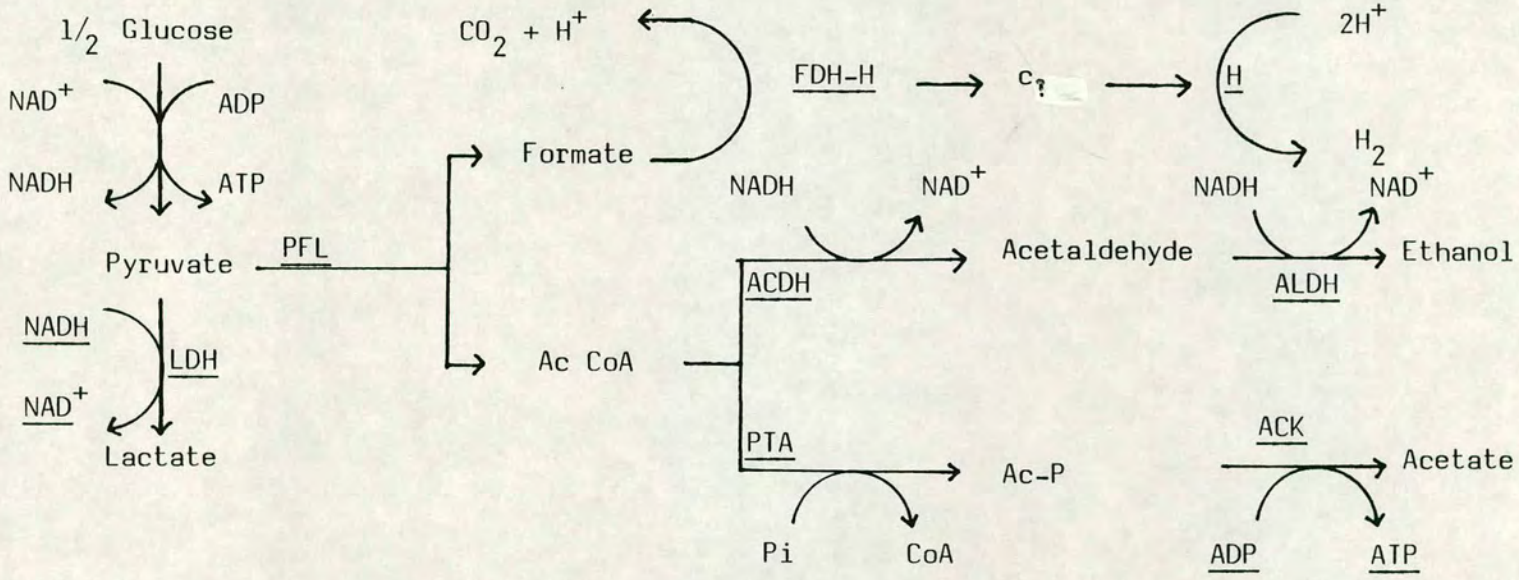
1.4 Fermentation

As a consequence of supporting NAD^+ regeneration, fermentation (substrate level phosphorylation) permits ATP synthesis by glycolysis (Figure 1.4). In *E. coli* NAD^+ may be regenerated by either reduction of pyruvate to lactate (Wood, 1961) or by pyruvate-formate lyase activity. The latter converts pyruvate to formate and acetyl-CoA which is then further metabolised, in two separate pathways, to acetate (generating ATP) and ethanol (regenerating NAD^+) (Brown *et al*, 1977; Pascal *et al*, 1982). This mixed acid fermentation is characteristic of most enteric bacteria with acetate formation the most favourable pathway producing 1 mol ATP/mol acetate. Additional fermentation pathways such as that producing 2-3 butane-diol are utilised by *K. pneumoniae* in the presence of glucose (Taxeira de Mattos *et al*, 1984). These additional pathways are considered to allow for modulation of ATP synthase relative to glucose uptake, enabling rapid catabolism of glucose without affecting the anabolic capacity of the cell. However, fermentation is relatively inefficient in terms of ATP generation as it results in the accumulation of oxidised/reduced substrates. Furthermore, the oxidation/reduction balance can only be maintained if the overall oxidation level of the products are identical to the substrate, a restriction which limits substrate choice.

1.5 Anaerobic respiration

Anaerobic respiration generates energy by the coupling of substrate oxidation to the reduction of electron acceptors such as nitrate, fumarate, TMAO, sulphur, nitrogen and carbon oxides. The availability of compounds such as nitrate, fumarate and TMAO suppresses fermentative pathways, with evidence suggesting that nitrate regulates fermentative enzymes at the transcriptional level (Clark and Cronan, 1980). Other modes of anaerobic respiration involving oxides of sulphur, nitrogen, carbon and various metals are performed by specialist groups of bacteria: denitrifiers, methanogens, sulphate reducers and

Figure 1.4 Mixed acid fermentation



LDH: lactate dehydrogenase; PFL: pyruvate formate lyase; FDH-H: formate dehydrogenase-H; ACDH: acetaldehyde dehydrogenase; H: hydrogenase; ALDH: alcohol dehydrogenase; PTA: phosphotransacetylase; ACK: acetate kinase; Ac-CoA: acetyl Coenzyme A.

iron oxidisers. As these respirations are not directly relevant to this thesis, I will only briefly mention them here. For reviews, refer to the following literature (Moodie and Ingledew, 1990; Cole and Ferguson, 1988; Odom and Peck, 1984). Respiratory chains involving nitrate, fumarate and TMAO reductases are more widespread among Gram negative bacteria and are discussed in detail in the following sections.

1.6 Nitrate reductase

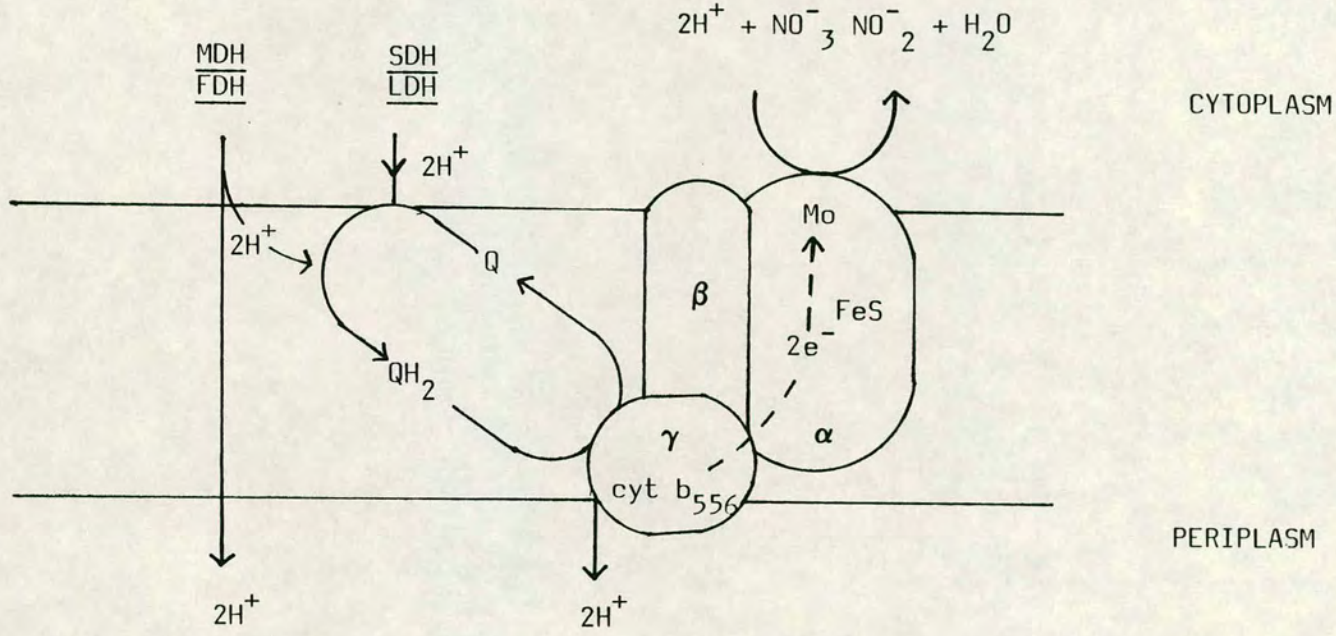
Distinct nitrate reductases have been identified in *Ps. aeruginosa*, *K. pneumoniae* and *S. typhimurium* (Jeter *et al*, 1984; Stewart, 1988; Barrett and Riggs, 1982), which fall into two categories. The first, known as assimilatory nitrate reductase allows aerobic incorporation of nitrogen for biosynthetic purposes. The second, supports anaerobic nitrate respiration and is common to many other bacteria, including *E. coli*.

Respiratory nitrate reductase of *E. coli* is a molybdoenzyme which accepts electrons from substrate oxidation to reduce nitrate (NO_3^-) to nitrite (NO_2^-) with energy conserved as a proton motive force for ATP generation. The proton motive force is generated through the release of two protons in the periplasm, by oxidation of formate coupled to the reduction of quinol, and the consumption of two protons in the cytoplasm by the reduction of nitrate (Figure 1.5). Nitrite (in the *Enterobacteriaceae*) can be reduced to ammonia thus regenerating oxidised nicotinamide cofactor by a six electron transfer or can form a proton motive force (Boonstra *et al*, 1975; Konings and Kaback, 1973).

1.6.1 Components of nitrate respiratory chain

Nitrate reduction in *E. coli* can be coupled to the oxidation of either formate, glycerol-3-phosphate, hydrogen, D-lactate, malate, NADH or succinate.

Figure 1.5 Nitrate reduction in *E. coli*



Substrate oxidation is coupled to quinol reduction. In turn this is coupled to nitrate reduction via electron transfer through the α subunit.

MDH: malate dehydrogenase; FDH: formate dehydrogenase; SDH: succinate dehydrogenase; LDH: lactate dehydrogenase; Q: quinone; Mo: molybdenum cofactor.

Intermediate components have been identified in the nitrate reductase respiratory pathway. Quinones are essential for electron transfer from substrate to terminal reductase. Using isogenic *ubi*A (defective in ubiquinone production) and *men*A (defective in production of menaquinone) mutants of *E. coli*, Wallace and Young (1977a,b) showed that anaerobic growth was severely depressed in the *ubi*A strain but not in the *men*A strain and that high levels of menaquinone were induced during anaerobic growth with nitrate. This implicated the preferential use of ubiquinone in aerobic respiration and menaquinone in anaerobic respiration. In a double *men*A *ubi*A mutant, the fact that NADH oxidation coupled to nitrate reduction was restored by addition of ubiquinone and glycerol-3-phosphate, and formate-nitrate reduction was restored by either ubiquinone or menaquinone, indicated that both NADH and formate could be used simultaneously and independently as electron donors for nitrate reduction. This utilisation of two distinct quinones was considered to be due to various factors: relative redox potentials enabling certain respiratory chains to work more efficiently with a particular quinone, or regulation of *in vivo* activity of various respiratory chains where more than one electron acceptor of differing potential are present or different dehydrogenases may recognise different structural features in quinones.

Cytochrome b was implicated in nitrate respiration by Ruiz-Herrera and DeMoss (1969), who isolated *E. coli* mutants defective in nitrate respiration and found that many had lowered levels of cytochrome b. Furthermore, cytochrome b spectra were similar for both wild-type and mutant cells cultured anaerobically indicating aerobic and anaerobic cytochrome b species to be genetically distinct (Ruiz-Herrera, *et al*, 1969). A single α-band absorption at 556nm was determined, but kinetics showed two distinct b cytochromes in the formate-nitrate respiratory chain, and one, presumably the formate dehydrogenase specific cytochrome, inferred to be very electronegative (Sanchez-Crispin *et al*, 1979).

1.6.2 Structure of nitrate reductase

Nitrate reductase was purified from *E. coli* envelope fractions and shown to consist of two subunits, one of 150 kDa (α) and one of 60 kDa (β) present in a 1:1 ratio. A third subunit of approximately 20 kDa (γ) was found to be associated with the enzyme (Enoch and Lester, 1974, 1975). This subunit was analysed by physical and spectral studies and found to be the nitrate reductase-specific cytochrome b_{556} (Chaudhry and MacGregor, 1983). The nitrate reductase from the denitrifying bacterium *Paracocus denitrificans* is strikingly similar to that of *E. coli* with molecular weights 127 kDa, 61 kDa, and 21 kDa for the α, β and γ subunits respectively. As in *E. coli*, the subunits are found in the ratio 2:2:4 (Craske and Ferguson, 1986). There may be a second b-type cytochrome in the γ subunit, suggested following the observation implicating two acceptor groups for the two-electron oxidation of quinol (Ballard and Ferguson, 1987). The nitrate reductase from other denitrifiers such as *Pseudomonas aeruginosa* has been shown to possess α and β subunits (Carlson *et al*, 1982), but a γ subunit was not detected, possibly because it readily dissociates from the enzyme during purification. The nitrate reductase α, β, and γ structural subunits were originally thought to be encoded from a single operon termed *nar*: *narC* encoding the α subunit, *narH* the β subunit and *narI* the γ subunit. Recent work has shown the *narI* locus to contain two genes encoding proteins of 26.5 kDa and 25.5 kDa (Sodergren and DeMoss, 1988), which were subsequently designated NarJ and NarI. The γ subunit of nitrate reductase was suggested to be encoded by *narI* (the gene product of which was hydrophobic and thus compatible with the proposed γ subunit function as membrane anchor for nitrate reductase). The function of the *narJ* gene product was not fully resolved, but considered to be involved either with mediating transfer of haem into the nitrate reductase complex or facilitating binding of the $\alpha\beta$ complex to the γ subunit. Additional genes in the *nar* locus include *narK, narL* and *narX*. As yet, no function has been

assigned to *narK* althougth it has been implicated in nitrate transport (Stewart and Parales, 1988). The *narL* and *narX* gene products are involved in regulatory control of anaerobic respiratory enzymes and are discussed more fully in Section 1.9.2.

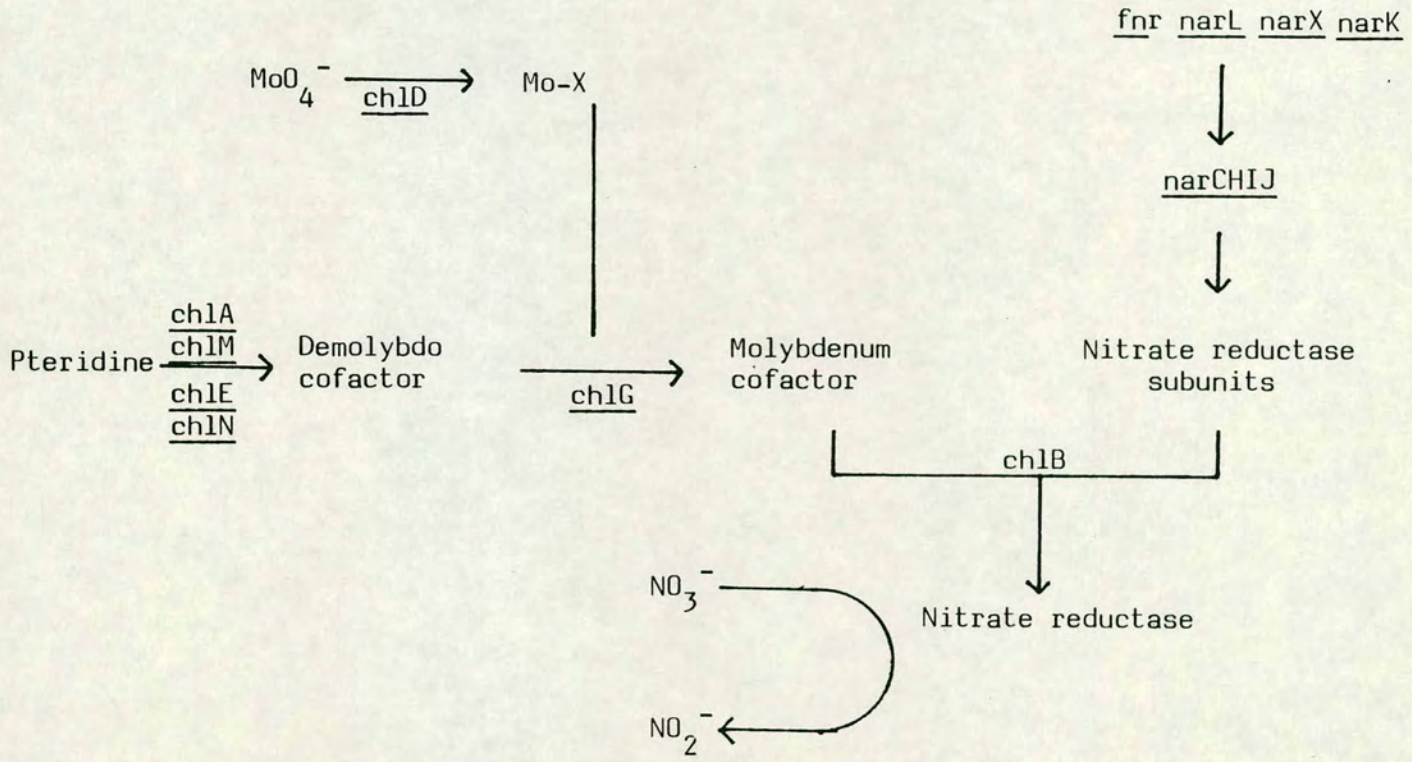
Nitrate reductase purified from *K. pneumoniae* S45 (van't Riet and Planta, 1975) and *Proteus mirabilis* DG (Oltmann *et al*, 1976) was found to be composed of three subunits with none of the subunits a cytochrome specifically associated with the enzyme, in contrast to the *E. coli* nitrate reductase. However, the α and β subunits of *E. coli* K12 and *K. pneumoniae* were considered to have some homologous regions, being immunologically cross reactive (Abraham *et al*, 1981).

The *E. coli* α subunit was considered to be the active site for nitrate reduction as enzyme activity was lost during conditions which remove molybdate from the α subunit and reconstituted by its replacement. Molybdate is present in nitrate reductase as Mo cofactor (Amy and Rajagopalan, 1979) present at approximately one complete molecule per $\alpha\beta$ monomer (Adams and Mortensen, 1983) and bound to molybdopterin, in which form it is protected from inactivation by heat and oxygen. As with *E. coli*, the α-subunit of *P. denitrificans* contains molybdate. The proteins in *E. coli* thought to be involved with Mo cofactor synthesis were the products of gene loci designated *chl* with nitrate reductase mutants selected as being resistant to chlorate, chlorate being a substrate for nitrate reductase. Further early work suggested that *chl* gene products were involved in Mo cofactor synthesis: (1) high concentrations of Mo phenotypically suppressed *chlD* mutants (Glaser and DeMoss, 1971; Sperl and DeMoss, 1975), (2) certain *E. coli* mutants could not provide a source of Mo cofactor for reconstitution of nitrate reductase activity (MacGregor and Schnaitman, 1973), and (3) the Mo labelling studies showing that *chl* mutants could not incorporate Mo into nitrate reductase (Dubourdieu *et al*, 1976; Grassi *et al*, 1979). It had previously been discovered (Azoulay *et al*, 1967) that nitrate

reductase activity was restored on mixing cell free extracts from *chlA* and *chlB* mutants. Furthermore, purified *chlA* and *chlB* gene products reconstituted the *in vitro* activities of the molybdoenzymes formate dehydrogenase and TMAO reductase (Grillet and Giordono, 1983; Riviere *et al*, 1975). The *chlA* locus was suggested to be involved in Mo cofactor synthesis and the *chlB* gene product to mediate the incorporation of Mo cofactor into apo-respiratory nitrate reductase (MacGregor and Schnaitman, 1973). Other *chl* loci have since been identified. The gene products from *chlA* (*chlA* and *chlM*) and *chlE* (*chlE* and *chlN)* are involved in biosynthesis of the demolybdo cofactor (Johnson and Rajagopalan, 1987) with the *chlD* gene product catalysing either the transport or processing of molybdate into an intermediate MoX (Ugalde *et al*, 1985). The next step, the formation of Mo-cofactor from MoX and the demolybdo cofactor is believed to be catalysed by the *chlG* gene product (Miller and Amy, 1983), with the final incorporation of Mo-cofactor into molybdo proteins catalysed by the *chlB* gene product (MacGregor, 1975). This biosynthetic pathway is shown in Figure 1.6. Mutations in the *chl* gene loci can impose a more complete block of nitrate reduction than mutations only affecting the *nar* operon due to a secondary respiratory nitrate reductase also dependent on the Mo-cofactor (Lin and Kuritzkes, 1987).

Each $\alpha\beta$ monomer of nitrate reductase contains 3-4 $[4Fe\text{-}4S]^{2+,1+}$ clusters (Johnson *et al*, 1985) although active enzyme may also contain a 3Fe cluster similar to that found in bacterial ferredoxins, resulting from degradation of a 4Fe-4S cluster during enzyme isolation. The β subunit is possibly involved in membrane association and may also have a role in mediating subunit interactions (Stewart, 1988). The γ subunit is essential for enzyme incorporation into the cytoplasmic membrane as illustrated by Stewart and MacGregor, 1982, who showed that a mutant lacking the γ subunit accumulated nitrate reductase α and β subunits in the cytoplasmic fraction (Stewart and MacGregor, 1982). Current evidence suggests that the structure of native nitrate reductase is $2\alpha 2\beta 4\gamma$

Figure 1.6 Synthesis of molybdenum cofactor and insertion into respiratory nitrate reductase



(Chaudry and MacGregor, 1983) and the enzyme has a transmembrane orientation enabling it to generate a proton motive force (as previously described) with the α and β subunits exposed on the cytoplasmic face and the γ subunits on the periplasmic aspect of the cytoplasmic membrane (Graham and Boxer, 1980; Boxer and Clegg, 1975; MacGregor and Christopher, 1978).

1.7 Fumarate reductase

Confirmation of a fumarate reductase in *E. coli* that was distinct from succinate dehydrogenase was first discovered by Hirsch who found that anaerobic fumarate reduction was unimpaired in a mutant lacking succinate dehydrogenase activity. Although fumarate reductase is able to oxidise succinate, and succinate dehydrogenase can reduce fumarate, they do so with different rates and substrate affinities (Hirsch *et al*, 1963; Dickie and Weiner, 1979), with fumarate reductase having a greater affinity for fumarate than succinate and vice versa.

1.7.1 Structure of fumarate reductase

In *E. coli*, the fumarate reductase and succinate dehydrogenase are structurally very similar, both being flavoproteins with FAD as the prosthetic group, an iron sulphur protein, and are bound to the inner aspect of the cytoplasmic membrane by two anchor proteins, (Ingledew and Poole, 1984). This structural similarity coupled to their ability to catalyse the same reactions suggests that they may have evolved from gene duplication, with the succinate dehydrogenase being active aerobically (Hirsch *et al*, 1963), and fumarate reductase repressed in the presence of oxygen and nitrate but induced anaerobically in the presence of fumarate (Spencer and Guest, 1973).

Fumarate reductase mutants were first isolated by Lambden and Guest (1976) following mutation with nitrosoguanidine and selection for the inability of colonies to grow anaerobically with fumarate as sole electron acceptor.

Complementation of the mutants with an *E. coli* plasmid gene bank (Clarke and Carbon, 1976) identified two hybrid plasmids capable of reversing the mutant phenotype (Loheimer *et al*, 1981). Purification of the plasmid encoded gene product yielded a 95 kDa enzyme composed of two subunits of 69 kDa and 27 kDa from the same operon, which was subsequently designated *frd*. The *frdA* gene product (the 69 kDa subunit) contained a catalytically essential sulphydryl group (Robinson and Weiner, 1982) and a covalently bound 8α(N3-histidyl) FAD cofactor (Weiner and Dickie, 1979). The 27 kD β subunit (frdB) contained three Fe-S centres (Cammack *et al*, 1986; Cole *et al*, 1982, 1985) identified by EPR as a two-iron (2Fe-2S) ferredoxin centre with E_{m7} of -50 mV (Ingledew, 1983); a four-iron (4Fe-4S) ferredoxin centre with an E_{m7} of -285 mV (Cammack *et al*, 1986), and a three iron (3Fe-4S) centre with an E_{m7} of -50mV (Morningstar *et al*, 1985; Simpkin and Ingledew, 1985). The *frdA* and *frdB* subunits were present in a 1:1 molar ratio, comprising a catalytically active enzyme extending into the cytoplasm and bound to the inner aspect of the cytoplasmic membrane (Dickie and Weiner, 1979). Two additional genes were discovered directly downstream of *frdB* in the *frd* operon. These genes encoded polypeptides of 15 kDa (*frdC*) and 13 kDa daltons (*frdD*). These subunits were hydrophobic and involved in membrane attachment of the two larger polypeptides (Lemire *et al*, 1982; Gundstrom and Jaurin, 1982), in addition to which important in maintaining enzyme structure and allowing enzyme interaction with quinones (Lemire *et al*, 1982; Ceccini *et al*, 1986).

1.7.2 Components of fumarate respiratory chain

Formate, NADH and glycerol-3-phosphate dehydrogenase all support the reduction of fumarate (Ingledew and Poole, 1984). The ability of haem deficient mutants to grow anaerobically on glycerol or glycerol-3-phosphate with fumarate, indicated that cytochromes were not involved in electron transport to fumarate (Singh and Bragg, 1975) although fumarate-dependent active transport

processes could not be demonstrated in the cytochrome-deficient cells suggesting that cytochrome b was responsible for coupling electron to proton translocation, with menaquinone linking the dehydrogenases to the cytochromes.

The reduction of fumarate is common among facultative and obligate anaerobes such as *V. succinogenes*. A DNA probe of the highly conserved *E. coli frdA* FAD-binding region hydridised strongly to chromosomal digests from various members of the *Enterobacteriaceae* (Unden and Cole, 1983). No hydridisation was detected to DNA from *V. succinogenes* which was surprising considering the strong structural resemblance of the *E. coli* and *V. succinogens* fumarate reductases. The *V. succinogenes* enzyme is composed of two catalytic subunits (Unden *et al*, 1980) linked to cytochrome b in the ratio 1:1:2, with the cytochrome b anchoring the enzyme to the cytoplasmic membrane. Similarly to the *E. coli* fumarate reductase FAD was covalently bound to the large (79 kDa) subunit, and the enzyme was partially reversible catalysing a low level of succinate oxidation. The lack of homology with the *E. coli* enzyme, despite similarities of structure and amino acid composition of the catalytic subunits, implied that the two genes were a product of convergent evolution.

Fumarate reductases have been isolated from other organisms such as the sulphate-reducing bacterium *Desulfovibrio multispirans* (He *et al*, 1986) and baker's yeast (Moratsubaki and Katsume, 1982). These enzymes differed from those of *E. coli* and *V. succinogenes* in catalysing unidirectional fumarate reduction, with non-covalently bound FAD as prosthetic group. The enzyme from *D. multispirans* was structurally similar to those of *E. coli* and *V. succinogenes*, comprising four subunits of 45 kDa, 32 kDa, 30 kDa and 27 kDa bound to the inner aspect of the cytoplasmic membrane. That of baker's yeast was cytosolic and structurally quite different being a single polypeptide of 58.8 kDa and containing no non-haem iron.

1.8 TMAO reductase

The involvement of bacteria in the reduction of TMAO was first demonstrated in 1937 by Beatty and Gibbons where they showed that the appearance of TMA, and increased bacterial counts during the storage of fish correlated with a decrease of TMAO, implying that TMAO reduction supported the reduction of bacterial respiration. TMAO reduction during oxygen limitation and anaerobiosis has been reported in a variety of different bacteria. They include *Shewanella*, (or *Alteromonas*), *Rhodopseudomonas capsulata*, *Proteus vulgaris*, *Vibrio parahaemolyticus*, *Achromobacter spp.*, *Photobacterium*, various members of the *Enterobacteriaceae* and possibly enteric *Campylobacter* (reviewed in Kwan and Barrett, 1985).

1.8.1 Structure of TMAO reductase

TMAO reductases have been isolated and purified from both *E. coli* and *S. typhimurium*. *E. coli* has one constitutive TMAO reductase of 100 kDa and 3 inducible enzymes (encoded from the *tor* and *dms* loci, see Section 1.8.2), the most active of which is a membrane-bound molybdoenzyme of 220 kDa daltons and composed of two 80 kDa and 60 kDa subunits (Shimokawa and Ishimoto, 1979). Similarly to *E. coli, S. typhimurium* produced a constitutive TMAO reductase of 375 kDa, a major inducible enzyme with a native molecular weight of 332 kDa being a tetramer of an 84 kDa subunit, and a further one or possibly two minor enzymes (Kwan and Barrett, 1983). TMAO reductase synthesis in both *E. coli* and *S. typhimurium* required molybdenum in the growth medium (Takagai *et al*, 1981). The absence of enzyme activity in *chlD* mutants (Cox and Knight, 1981) which was restored by addition of molybdenum to the growth medium strongly suggested that the TMAO reductases were molybdoenzymes.

TMAO reductases are found either as integral membrane proteins or loosely bound and easily released into the periplasmic cell fraction. The enzymes in the former category include those from *S. typhimurium* (Kwan and

Barrett, 1983), those from *V. parahaemolyticus* (Unemoto *et al*, 1965) and the major inducible 200 kDa and constitutive 70 kDa enzymes from *E. coli*. Those in the latter category include the inducible 100 kDa and second 70 kDa enzyme from *E. coli*.

1.8.2 Components of TMAO respiratory pathway

TMAO was shown to support anaerobic respiration during translocation, active transport and oxidative phosphorylation studies using membrane vesicles derived from *E. coli* (Takagi and Ishimoto, 1983). Formate, NADH and lactate were all shown to support TMAO reduction in *E. coli* (Sakaguchi and Kawai, 1977; Yamamoto and Ishimoto, 1977; Strom and Larsen, 1979; Strom *et al*, 1979; Nishimura *et al*, 1983). Lactate was implicated as the natural donor for TMAO reduction during fish spoilage as its high concentration in fish muscle decreases during TMAO reduction (Strom and Larsen, 1979; Strom *et al*, 1979).

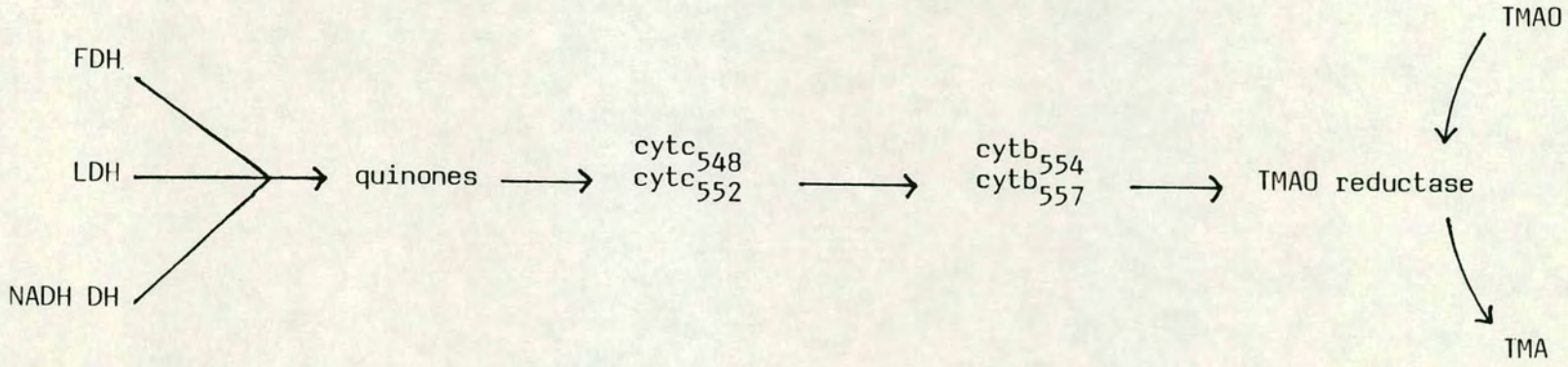
The existence of cytochromes as intermediary electron carriers in the reduction of TMAO by *E. coli* was confirmed by Cox and Knight (1981) who found that mutants defective in cytochrome biosynthesis were incapable of respiration with TMAO. It was suggested that various b and c type cytochromes were involved in the reduction of TMAO in *E. coli* (Sakaguchi and Kawaii, 1977), the b type cytochromes being constitutive and the c type cytochromes induced by the presence of TMAO under anaerobic conditions (Sakaguchi *et al*, 1979), although these studies were confused by the presence of other cytochromes involved in separate respiratory pathways. Bragg and Hackett (1983) discovered three groups of cytochromes in cells of *E. coli* grown anaerobically with TMAO. Fifty percent were involved in aerobic respiration, 25% were not involved in either aerobic or anaerobic respiration: the remainder, two b and two c type cytochromes, were considered to be involved in the reduction of TMAO as they were rapidly reoxidised by TMAO in contrast to

those of aerobic respiratory pathways. These cytochromes were also reoxidised by the membrane impermeable oxidant ammonium persulphate indicating that cytochromes from the TMAO reductase pathway were sited at the periplasmic aspect of the cytoplasmic membrane (Bragg and Hackett, 1983). Copper sulphate inhibited the reduction of TMAO by formate, preventing reduction of the c-type cytochromes c-548 and c-552, but having no corresponding effect on the b-554 and b-557 cytochromes implying that the c type cytochromes were situated prior to the b-type cytochromes in the respiratory chain (Figure 1.7). Determination of the electropotential of the respective cytochromes to corroborate the proposed sequence was attempted but proved inconclusive. In *Proteus spp.* b-type but not c-type cytochromes are involved in TMAO respiration (Takagi *et al*, 1981).

A possible role for quinone as the immediate electron donor for the c-type cytochromes in *E. coli* was suggested (Bragg and Hackett, 1983). Menaquinone was proposed as the immediate electron donor for the cytochrome of the *E. coli* TMAO pathway, since a mutant lacking menaquinone had lost NADH-TMAO reductase activity (Cox and Knight, 1981). Menaquinone is also essential for TMAO reductase activity in *S. typhimurium*, in contrast to *E. coli*, where ubiquinone can substitute for menaquinone.

A membrane bound terminal reductase was cloned from *E. coli* which could reduce TMAO, DMSO and methionine sulphoxide (Bilous and Weiner, 1988). This enzyme was genetically distinct from the genetic loci identified for TMAO reductase (*tor*), as three polypeptides were identified from the cloned gene which agreed with the subunits associated with purified DMSO reductase (Weiner *et al*, 1988) encoded by the *tor* operon. TMAO and DMSO are reduced by the same enzyme in *Rhodobacter capsulatus* (McEwan *et al*, 1987) and *Proteus vulgaris* (Strudd and Strom, 1984). This interaction of several different compounds with the same enzyme is not unusual and may be further evidence supporting metabolic diversity of bacteria.

Figure 1.7 *E. coli* TMAO reductase pathway



FDH: Formate dehydrogenase

LDH: Lactate dehydrogenase

NADH DH: Nictoine adenine dinucleotide dehydrogenase

1.9 Regulation of terminal reductase expression

Anaerobic generation of ATP discussed so far has shown that energy can be obtained via oxidative phosporylation by the reduction of a variety of terminal electron acceptors. During normal bacterial growth, preference is given to the terminal electron acceptor with the highest redox potential allowing maximum ATP generation. Therefore in *E. coli* oxygen (E'_0 = 0.82 V) is used in preference to nitrate, TMAO and fumarate, the reductases of which are repressed aerobically and nitrate E'_0 (0.42) used preferentially to fumarate (E'_0 = 0.031 V) and TMAO (E'_0 = 0.13 V). The regulation of anaerobic respiratory systems is discussed below.

1.9.1 Regulation by arcA, arcB and fnr

Factors involving the co-ordination of gene expression in response to changes in oxygen tension have been extensively studied. In *E. coli*, two global transcriptional regulators have been identified. One, the two component sensor-regulator (Arc B-A), represses a wide variety of aerobic enzymes under anaerobic conditions (Iuchi and Lin, 1988, 1989; and reviewed by Spiro and Guest, 1991). The other, Fnr, is essential for activating the expression of anaerobic respiratory processes (reviewed by Spiro and Guest, 1990).

The gene-derived primary structures of ArcA and ArcB (Iuchi *et al*, 1990a) show that they belong to the family of two-component signal-transducing systems which mediate a wide range of adaptive responses in bacteria (Stock *et al*, 1989). This suggests ArcA is a cytoplasmic regulator with a putative DNA-binding domain, and ArcB a membrane-bound sensor (Iuchi *et al*, 1990b). It would appear that anoxia-induced changes in the ratio of oxidised to reduced forms of an electron carrier, either located in or directly coupled to the electron transport chain, is detected by ArcB (Iuchi *et al*, 1990). ArcB thus becomes autophosphorylated in response to oxygen limitation, transmitting the signal to

ArcA by converting it into an active phosphorylated form where it represses aerobic enzymes such as TCA cycle enzymes, pyruvate, L-lactate, D-amino acid and 3-Hydroxyl-CoA dehydrogenase and the low affinity cytochrome *o* oxidase whilst inducing the high affinity cytochrome *d* oxidase.

Several research groups independently reported a single mutation in *E. coli* termed *fnr* (Lambden and Guest, 1976), *nirA* (Cole and Ward, 1973; Newman and Cole, 1978), and *nirR* (Chippaux *et al*, 1978), the effects of which were pleiotropic causing deficiencies of many anaerobically induced enzymes including nitrate and fumarate reductases, glycerol-3-phosphate dehydrogenase and formate dehydrogenase (Jamieson *et al*, 1986; Kuritzkes *et al*, 1984). It was later proposed that *fnr*, *nirA*, and *nirR* corresponded to the same gene and that the *fnr* gene product was a positively-acting regulatory factor. The *fnr* gene was cloned, sequenced and the protein product identified (Shaw and Guest, 1981, 1982a, b) as a protein of slightly less than 28 kDa whose primary structure contained a region homologous with several transcriptional regulator proteins. The *fnr* gene product was expressed both aerobically and anaerobically (Spiro and Guest, 1987) with expression suppressed approximately 2.7 fold during aerobic conditions. This expression was dependent on the presence of Fnr protein, implying that during anaerobiosis *fnr* was able to adopt an active configuration where it could both self regulate and activate *fnr*-dependent genes. A DNA sequence previously identified in promoter or transcription initiation sites of several genes under *fnr* control was found to be similar to that of hyphenated dyad symmetry identified in the 5' non-coding region of the *fnr* gene. This sequence was located downstream of the *fnr* promoter and as such consistent with the observed autoregulation of Fnr, strongly suggesting this conserved DNA sequence to be the Fnr binding site. Comparison of nucleotide sequences from the promoter region of many genes under Fnr control has identified a putative consensus sequence for the Fnr-binding site (Eiglmeier *et al*, 1989; Spiro and Guest, 1987; Jayaraman *et al*, 1989). To date, in *E. coli*

more than 20 genes with this consensus sequence, thus regulated by Fnr, have been identified (see Spiro and Guest, 1990).

Amino acid sequence homology between Fnr and the cyclic AMP receptor protein Crp, a DNA binding protein which mediates catabolite repression at transcriptional level (De Combrugghe *et al*, 1984; Busby, 1986) strongly indicated that Fnr functioned as a transcriptional regulator (Shaw *et al*, 1983). Homology between the amino acid sequences of the two proteins was particularly striking in the DNA-binding domain (Shaw *et al*, 1983). Crp and Fnr recognise similar binding sites, each a 22 base pair sequence containing partial two-fold rotational symmetry (Spiro and Guest, 1987; Jayaraman *et al*, 1989, Eiglmeier *et al*, 1989).

Other proteins showing a high degree of sequence similarity, indicating the presence of analogous DNA and nucleotide-binding sites, with Fnr have recently been identified: the Fix K protein from *Rhizobium meliloti* which functions both positively and negatively in the regulatory cascade controlling nitrogen fixation (Batut *et al*, 1989), HylX from *Actinobacillus pleuropneumoniae*, a haemolysin which may also regulate haemolysin synthesis (MacInnes *et al*, 1990) and FnrN from *Rhizobium leguminosarum* which regulates microaerobic nitrogen fixation (Colonna *et al*, 1990).

The presence of a cysteine cluster in Fnr (but not in Crp) which was essential for complete functional activity (Spiro and Guest, 1988) suggested that a nucleotide co-effector was involved in signalling anaerobiosis. This co-effector was not cAMP (Unden and Duchene, 1987 which was previously suggested as an Fnr co-effector). Circumstantial evidence summarized by Spiro and Guest (1990), suggested that the regulatory function may be directly related to the anaerobic incorporation of a metal ion which reduces the accessibility of the cysteines and activates Fnr. This dependence of activity on a metal ion links Fnr to other oxygen-sensing proteins such as the *Klebsiella pneumoniae* NifL protein, which regulates NifA, the transcriptional activator for *nif* genes (Henderson *et*

al, 1989), and the metal-activated ferric uptake protein (*Fur*) of *E. coli* (Bagg and Nielands, 1987). These proteins have a cysteine cluster similar to that of Fnr, for which a metal ion is essential for activity. The identification of many Fnr homologues in diverse microorganisms points to the importance of this family of regulatory proteins.

The role of Fnr in the regulation of nitrate, fumarate and TMAO reductases is discussed in the following sections.

1.9.2 Regulation of nitrate reductase expression

Other factors in addition to Fnr and anaerobiosis seem to be required for full induction of nitrate reductase; these factors also repress transcription of the fumarate reductase operon, and presumably also other terminal reductases. It was shown by Iuchi and Lin (1987), that complete induction of nitrate reductase and complete repression of fumarate reductase were effected by the *narL* gene product, thought to be molybdate. Furthermore, mutants deficient in the transport of molybdate did not induce nitrate reductase or repress fumarate reductase unless molybdate was added to the growth medium. Thus, when nitrate reductase can be produced in a catalytically active form, transcription of other terminal reductases is prevented. The *narL* locus (also identified as *frdR* (Kalman and Gunsalus, 1988) has recently been discovered to encode two gene products which were termed *narL* and *narX* (Kalman and Gunsalus, 1989). Mutants of either *narL* or *narX* were pleiotropic depressing *frd* and TMAO expression and causing defective induction of nitrate reductase in cells grown in the presence of nitrate. This indicated that *narL, narX* and *fnr* gene products were required for normal control of anaerobic electron transport operons.

Expression of *narL* was found not to be dependent on the *fnr* gene product (Stewart and Parales, 1988) whereas *narX* expression required both NarL and Fnr. The observations that NarX is not absolutely required for NarL to function as a transcriptional activator whereas mutations of *narX* almost

eliminate nitrate repression of *frdABCD* have led to the following suppositions: the *narL* gene product encodes a DNA-binding regulatory protein of 28 kDa, and *narX* either a nitrate sensor protein (of 66 kDa), or a product involved in converting *narL* to a repressor conformation (Stewart *et al*, 1989) where it can bind to DNA and modulate expression of anaerobic enzymes. In the absence of nitrate, NarX may also convert NarL to an inactive form which is unable to repress or activate gene expression. A secondary nitrate reductase has been characterised in *E. coli* (Iuchi *et al*, 1987). Enzyme activity is constitutive and independent of regulation by oxygen, nitrate or Fnr. This enzyme couples formate oxidation to nitrate reduction and requires molybdate for activity (See Section 1.6.2).

1.9.3 Regulation of fumarate reductase

The presence of oxygen, nitrate and fumarate affect the level of fumarate reductase in the cell. Fumarate alone induces a high level of fumarate reductase, with induction being mediated through *fnr*: fumarate induction of *frd* was lost in an *fnr* mutant and restored on addition of *fnr* in *trans* (Jones and Gunsalus, 1985).

Oxygen and nitrate reduced fumarate reductase expression (Jones and Gunsalus, 1985) regardless of fumarate concentration. The mechanism of nitrate repression appears to be independent of nitrate respiration and oxygen control imparted by *fnr*, as nitrate repressed fumarate reductase activity aerobically and anaerobically (Jones and Gunsalus, 1987). Anaerobic expression in an *fnr* mutant, however, was not as low as that observed in cells cultured aerobically. This was attributed either to residual *fnr* activity or to the presence of additional as yet unidentified regulatory proteins (Jones and Gunsalus, 1987). In *S. typhimurium* the anaerobic induction of *pepT* genes involves the loci *oxrA* and *oxrB* (Strauch *et al*, 1985). The *fnr* gene from *E. coli* can complement an *oxrA1* mutation in *S. typhimurium* (Jamieson and Higgins, 1984). This, in addition to

the fact that *oxrA* maps in a similar region to the *fnr* in *E. coli*, indicates a similarity between *oxrA* and *fnr*. A parallel to *oxrB* in *E. coli* has not yet been discovered. The protein encoded by the *oxrC* gene locus, mutations of which are highly pleiotropic although do not appear to affect the expression of *oxrA* dependent genes, affects stress-regulated gene exchanges via changes in the degree of DNA supercoiling (Ni Briain *et al*, 1989). It is likely that repression of the fumarate reductase operon in the presence of nitrate, avoids unnecessary synthesis of fumarate reductase during nitrate respiration.

1.9.4 Regulation of TMAO reductase expression

The same study showed that TMAO did not exert significant regulatory control over fumarate reductase gene expression (Jones and Gunsalus, 1985). This was rather surprising as the redox potential of TMAO (E'_0 = 0.13 V) is more positive than that of fumarate (E'_0 = 0.031 V). Regulatory elements for the preferential use of this terminal reductase apparently have not evolved in *E. coli*. Repression of TMAO reduction by nitrate was studied by Sakaguchi and Kawai (1975) who reported that nitrate repressed but did not inhibit TMAO reductase activity suggesting that control was exerted at the level of transcription.

In contrast, TMAO reduction appears to be significantly more important in the Gram negative bacterium *S. putrefaciens*.

1.10 TMAO reduction in *S. putrefaciens*

Two strains of *S. putrefaciens*: NCMB400 (Easter, 1982) and NCMB1735 (Ringo, 1984; Stenberg *et al*, 1984) were found to be capable of TMAO reduction when grown anaerobically using TMAO as a terminal electron acceptor. NCMB1735 was able to grow with formate as electron donor: formate and TMAO-dependent anaerobic uptake of serine was sensitive to ionophores in NCMB1735 (Stenberg *et al*, 1984) indicating anaerobic respiration involving an

ANAEROBIC RESPIRATION IN *Shewanella putrefaciens*

Shewanella putrefaciens (formerly *Alteromonas putrefaciens*) has a competitive advantage over many bacterial flora which colonise its native habitat, marine fish. This is due to it being able to respire when oxygen is limiting through its ability to utilise TMAO (a component of fish flesh and tissues) as a terminal electron acceptor. Anaerobic respiration is therefore of great importance to this organism and has been investigated by Dr Ward's research group since 1980.

It was found by Easter (1982), that anaerobic respiration in *S. putrefaciens* was supported by TMAO, nitrate and fumarate, with maximal induction of the components in each respiratory pathway being a combination of oxygen limitation and appropriate exogenous electron acceptor. Sub-cellular fractionation of oxygen-limited cultures indicated that TMAO, nitrate and fumarate reductases were found in the periplasmic cell fractions. Easter adapted the osmotic shock-lysis sphaeroplasting procedure of Birdsell and Cota-Robles (1967) to facilitate isolation of periplasm in *S. putrefaciens*. This method is currently used by Dr Ward's group as a reliable first stage in purification of periplasmic enzymes.

The TMAO reductase enzymes in *S. putrefaciens* were studied in detail by Clarke (1984). He isolated two TMAO reductases: one of 90 kDa, induced under microaerobic culture conditions by TMAO and DMSO, and one of 47 kDa synthesised constitutively under microaerobic conditions. Both enzymes were considered to be loosely associated with the outer surface of the cytoplasmic membrane being released into the periplasm upon sphaeroplasting. The enzymes were distinct from each other, as on purification to homogeneity, the 90 kDa enzyme was found to be a single polypeptide which did not dissociate into the second 47 kDa enzyme. Furthermore, polyclonal antibody raised to the purified 90 kDa enzyme showed that it was immunologically unrelated to the 47 kDa enzyme in *S. putrefaciens*, or to the TMAO reductase enzyme in *Escherichia coli* and *Salmonella typhimurium*. The presence of sulphydryl groups in the 90 kDa enzyme was suggested because it showed activity inhibition by thiol modifying agents. Clarke (1984) found that molybdenum was present in the 90 kDa enzyme, but not haem or non-haem iron, and although the absorption spectrum of purified enzyme suggested the presence of flavin, activity was not stimulated by the addition of exogenous flavin.

Regulation of anaerobic respiratory enzymes in *S. putrefaciens* was investigated by Nasser (1983). Confirming earlier work, the nitrate, fumarate and TMAO reductases were found to be predominantly periplasmic. Formate dehydrogenase was membrane bound. Neither nitrate nor formate in the growth media repressed the formate-TMAO respiratory pathway: cells grown on a combination of TMAO with either nitrate or formate had slightly enhanced TMAO reductase activity. Nitrate repressed formate reductase and vice-versa suggesting that they were controlled by the same regulatory system. Molybdate enhanced activities of the nitrate and TMAO reductases. The molybdenum cofactor from *S. putrefaciens* was similar to molybdenum cofactors from other sources in being inactivated upon exposure to oxygen. Molybdenum cofactor synthesis was induced by partial anaerobiosis when a considerable portion of free molybdenum cofactor was found in the periplasm.

Both Nasser (1983) and Lamont (1980) used ultraviolet or nitrosoguanidine mutagenesis to generate electron transport mutants of *S. putrefaciens*. In both cases a high percentage of the mutants were pleiotropic, all having lost TMAO reductase, nitrate reductase and formate dehydrogenase activities, but not formate reductase activity. This was considered to be due to

intact membrane. Other strains of *S. putrefaciens* also reduce TMAO, but have not been studied in detail.

1.10.1 Structure of TMAO reductase

Two distinct enzymes were identified from *S. putrefaciens* NCMB 400 by SDS PAGE and PAGE followed by zymogram staining for TMAO reductase activity (Clarke, 1984). One enzyme of 46 kDa was constitutive. The second enzyme (90 kDa) was induced to varying degrees by different culture conditions. High levels were induced anaerobically in the presence of TMAO. Much lower levels of the enzyme were detected in anaerobic cells with formate as terminal electron acceptor. The structure of TMAO reductase is discussed more fully in Section 1.10.3.

1.10.2 Regulation of TMAO reductase by nitrate, DMSO and pyridine N-oxide

Nitrate in the growth medium as sole terminal electron acceptor inhibited synthesis of the inducible TMAO reductase; however in the presence of both TMAO and nitrate there was no repression of TMAO reductase synthesis. This contrasts to the situation of *E. coli* (Section 1.8.4) where nitrate totally inhibits TMAO reductase synthesis regardless of the presence of TMAO. Therefore it suggests a different regulatory system in *S. putrefaciens*, with TMAO being preferred as an electron acceptor despite the lower redox potential of the TMAO/TMA couple (+0.13V) compared with the nitrate/nitrite couple (+0.42V). DMSO and pyridine N-oxide were effective inducers of TMAO reductase, inducing activity to 100% and 85% respectively of that induced by TMAO, but were gratuitous inducers only as they were found to be poor substrates for TMAO reductase (Clarke, 1984).

1.10.3 Characterisation of TMAO reductase

As with the TMAO reductase of *S. typhimurium*, FAD, FMN, cytochrome c, NAD(P)H and ascorbate were ineffective as electron donors (Clarke and Ward, 1987; Kwan and Barrett, 1983). This contrasts with *E. coli* where FAD, FMN, (Shimokawa and Ishimoto, 1979) and cytochrome c (Sagai and Ishimoto, 1973) were utilised as electron donors. The *S. putrefaciens* TMAO reductase was found to require molybdenum for activity. In contrast to all other investigated molybdoenzymes, which contain at least one other redox centre in addition to molybdenum (Spence, 1980), no flavin, haem or non-haem iron was found to be associated with the TMAO reductase of *S. putrefaciens*. In this respect the TMAO reductase of *S. putrefaciens* is unusual in that no other redox centres were detected.

The majority of TMAO reductase activity was found in the periplasm following sphaeroplasting using the lysozyme/osmotic shock procedure (Birdsell and Cota-Robles, 1967) unless EDTA was used in the preparation, disrupting the membrane releasing any remaining TMAO reductase (Easter *et al*, 1983). The TMAO reductase enzymes therefore were considered to be loosely attached to the outer aspect of the plasma membrane and easily released into the periplasm similarly to the TMAO reductases of both *E. coli* and *S. typhimurium*. The nitrate and fumarate reductase enzymes of *S. putrefaciens* are also periplasmic (Nasser, 1984) which directly contrasts with those of *E. coli* and *V. succinogenes*.

1.10.4 Analysis of cytochromes involved in TMAO reduction

Periplasmic and membrane associated cytochromes involved in the reduction of TMAO in *S. putrefaciens* were studied (Morris, 1987; Morris, Gibson and Ward, 1990). Four low-potential c-type cytochromes were identified by ion exchange chromatography, the most abundant of which, a flavocytochrome, was further purified and analysed. The molecular weight of this cytochrome was estimated as 84 kDa by SDS PAGE and 89 kDa by amino

acid analysis. It was confirmed as a single polypeptide being a single subunit when denatured with reducing agents and electrophoresed by SDS PAGE.

1.10.5 Characterisation of flavocytochrome c

Flavin was identified as FAD and its release from the protein under mild denaturing conditions indicated a non-covalent linkage. The presence of a flavin group in a c-type cytochrome is relatively unusual. Examples of c-type flavocytochromes with FAD as flavin prosthetic group have been found in various phototrophic bacteria. Flavocytochrome c from the purple sulphur bacterium *Chromatium vinosum* has a molecular weight of 72 kDa, consisting of a 46 kDa flavin subunit and either a 21 kDa di-haem subunit or two 10-15 kDa mono-haem subunits (Fukumori and Yamanaka, 1979; Yamanaka *et al*, 1979). In contrast flavocytochrome c from the green sulphur bacterium *Chlorobium thiosulfatophilum* has a 47 kDa flavin-subunit and a single mono-haem 10 kDa subunit (Yamanaka *et al*, 1979; Yamanaka, 1976). The flavocytochrome-c in these phototrophic bacteria is a sulphide:cytochrome c oxidoreductase which catalyses the oxidation of sulphide to elemental sulphur (Yamanaka and Kusai, 1976; Gray and Knaff, 1982).

Several species of *Pseudomonas* also contain a flavocytochrome c. Its $\alpha_2\beta_2$ structure is different to that of the flavocytochrome c in phototrophic bacteria, consisting of two identical flavin and two identical haem-containing subunits, of 48.6 kDa and 9.036 kDa respectively (McIntire *et al*, 1986). This flavocytochrome c is a *p*-cresol methylhydroxylase which catalyses the dehydrogenation and hydration of *p*-cresol, 4-ethyl and 4-n-propyl phenols to the corresponding alcohols (Reeve *et al*, 1988).

In contrast to flavocytochrome c from *S. putrefaciens*, the FAD flavin linkage with flavocytochrome c in both phototrophic bacteria and *Desulfovibrio* was covalent (Kenney *et al*, 1977; McIntire *et al*, 1981; Walker *et al*, 1974). Additionally, the midpoint redox potentials of flavocytochrome c from *S.*

putrefaciens at -204; -320 mV were substantially lower than that of the other flavocytochromes, being approximately -250 mV for *Pseudomonas* spp (Hopper, 1983), 98 mV in *Chlorobium* (Meyer *et al*, 1968) and 33 mV in *Chromatium* (Meyer *et al*, 1985).

The pyridine haemochrome system of flavocytochrome c from *S. putrefaciens* was typical for c-type cytochromes with two thioether bonds between the haem vinyls and the polypeptide chain. There was no evidence to suggest any non-standard linkage of haem, or the presence of other haem types. Redox titrations indicated that at least two potentially non-equivalent haems were contributing to the spectra. The flavocytochrome c spectrum was typical of class III cytochromes found in photosynthetic and sulphate reducing bacteria. Further haem analysis showed that flavocytochrome c was a hexahaem, with all the haems being of low redox potential, 50% of which bound CO. The flavocytochrome c protein was hydrophilic having a hydrophobicity of 0.89 kcal/mol protein^{-1} and a polarity index of 0.54 (the polarity indexes of hydrophilic proteins fall between 0.47 and 0.56). Due to the low redox potential of flavocytochrome c, it was proposed to occur early in the TMAO reductase pathway.

1.10.6 Location of flavocytochrome c

Although SDS PAGE haem staining showed that flavocytochrome c was present during aerobic growth, in comparison to other aerobic cytochromes the level was minimal. In contrast, during anaerobic growth flavocytochrome c was one of the most abundant cytochromes in extracts of *S. putrefaciens* cultured both in the presence and absence of exogenous nitrate, fumarate and TMAO. Flavocytochrome was found principally in the periplasmic fraction (in agreement with previous evidence showing that it was hydrophilic) with a small proportion associated with the membrane fraction. This was confirmed by EDTA treatment of membranes which significantly decreased the amount of bound cytochrome

suggesting that it was loosely attached to the outer aspect of the cytoplasmic membrane (Morris, 1987).

1.10.7 Proposed function of flavocytochrome c

Amino acid analysis showed that the composition of flavocytochrome c was similar to that of the hexahaem c552 nitrite reductase of *E. coli* (Anraku *et al*, 1986). No nitrite reductase activity was associated with flavocytochrome c or the enzyme activities associated with flavocytochromes from other organisms, nor did it react with the terminal reductases TMAO, DMSO or nitrate.

A role suggested for the flavocytochrome regarding its low redox potential and cellular location (associated with the periplasmic face of the cytoplasmic memebrane) was as a substitute for b-type cytochromes which are usually found associated with formate dehydrogenase complexes and accept electrons directly from formate.

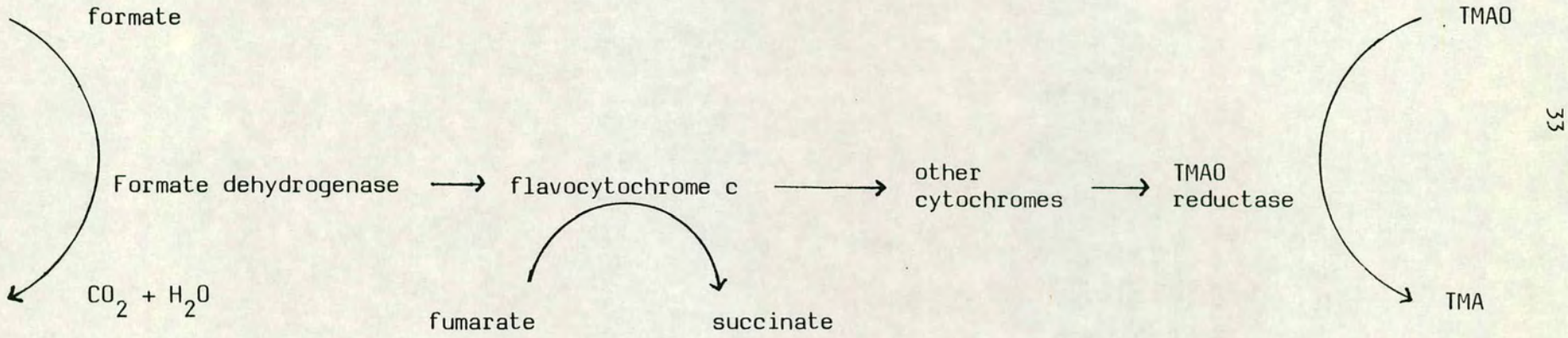
In a separate project which was attempting to purify the fumarate reductase from *S. putrefaciens* it was discovered that fumarate reductase activity was associated with the flavocytochrome at all stages of the purification (Ward, unpublished observations). This would suggest a dual role for the flavocytochrome, where it is implicated both in the reduction of TMAO where it acts as an electron acceptor directly from formate, and as the terminal electron acceptor fumarate reductase (Figure 1.8).

It was decided to clone flavocytochrome c to allow further characterisation of the protein by DNA and amino acid sequence analysis.

1.11 Gene cloning

The basic principles for cloning a gene are similar for most organisms. They demand a source of DNA encoding the required gene (usually the genome), a method for generating random DNA fragments, a suitable vector, the transfer of recombinant vector into a host cell for library amplification and

Figure 1.8 Role of flavocytochrome c in TMAO reduction by *S. putrefaciens*



screening, and a method by which to identify the recombinant clone containing the required gene. These general procedures have been extensively reviewed and well illustrated in the publications by Maniatis (1989); Boulnois (1987) and Old and Primrose (1985).

The purpose of the work in this thesis was to clone the flavocytochrome c gene from *S. putrefaciens* (a genetically uncharacterised Gram-negative bacterium). The following section discusses particular cloning strategies by which this could be achieved. It concentrates on host and vector choice for construction and maintenance of a gene library, and techniques for recombinant identification.

1.12 Vectors

Cloning vectors are derived from replicons that are stably inherited in bacterial cells in an extra chromosomal state. They are based either on plasmids (Bolivar *et al*, 1977), bacteriophage (Murray, 1983), cosmid (Collins and Hohn, 1979) or phagemids (Brenner *et al*, 1982). All have several common features: the ability to be expressed in at least one host, one or more unique restriction sites into which foreign DNA can be ligated, and a phenotypic selection marker. An extensive selection of vectors are available for both prokaryotic and eukaryotic cloning (see Pouwels *et al* (1985).

1.12.1 Plasmid

Plasmids are covalently closed circular molecules of DNA capable of replication independently of the host chromosome. Occuring naturally in bacteria, they range from 1 kb to 200 kb in size and encode traits such as antibiotic resistance, colicin production, and heavy metal resistance which confer a selective advantage to the host. Small plasmids (< 10 kb) are usually multicopy (Sherratt, 1982), whereas larger plasmids will be present in fewer

numbers: perhaps only one or two per cell and are conjugative (Clarke and Warren, 1979).

The first plasmid vectors employed were the naturally occurring *E. coli* plasmids ColE1 and pSC101, but these were soon superseded by *in vitro* constructed derivatives such as pBR322 (Bolivar, 1978) which subsequently became the progenitor of many plasmid and plasmid-derived cloning vectors. Most plasmid vectors used for cloning are less than 10 kb, for ease of manipulation and for more efficient transformation. They are multicopy and often amplifiable. Both of these factors lead to an increased level of plasmid encoded protein in the cell which facilitates identification of the desired recombinant (see Sections 1.13 and 1.14). Conjugative vectors are also available, enabling cloning in non-transformable bacteria. Plasmid vectors suitable for cloning flavocytochrome c are discussed in later sections.

1.12.2 Bacteriophage

Bacteriophage lambda (λ), a linear duplex molecule of 48.5kb which infects *E. coli* has been extensively developed for use as a vector (Murray, 1983; Blattner, 1977). The discovery that wild-type lambda contained non-essential regions of DNA resulted in the construction of improved vectors with a greater cloning capacity. In the wild-type state only 5% additional DNA can be inserted into λ due to packaging constraints, making it relatively unsuitable as a cloning vector. The λ based vectors are categorised into two general types: replacement vectors and insertion vectors.

1.12.2.1 Replacement vectors

Replacement vectors such as λWES B' (Thomas *et al*, 1974; Leder *et al*, 1977) have been constructed so that removal of non-essential regions without insertion of foreign DNA results in a phage particle which is too small to be packaged. Only recombinant phage will yield viable phage particles, thus

selecting against non-recombinants. Relatively large DNA fragments of up to 25 kb can be cloned in these vectors.

1.12.2.2 Insertion vectors

These contain a single restriction site into which foreign DNA can be inserted thus increasing vector size. Either (as with replacement vectors) the bacteriophage is too small to be packaged without insertion of foreign DNA, or the cloning site is located in a gene encoding a selectable phenotype which positively selects for recombinants. Bacteriophage such as these include Charon16A (Blattner *et al*, 1977), λgt10 (Nathans and Hogness, 1983; Young and Davies, 1983) and λNM1149 (Murray, 1983).

1.12.3 Cosmid vectors

A cosmid is a plasmid-bacteriophage hybrid, carrying the plasmid origin of replication and the bacteriophage *cos* site which enables in-vitro packaging. An example of this vector type is the general purpose cosmid pC655EMBL (Ehrich *et al*, 1987). DNA is cloned into the cosmid vector in its plasmid state, packaged into phage and the host strain transfected with the cosmid library. Cosmid vectors have two main advantages (1) the large amount of foreign DNA which they can accommodate, approximately 39 kb to 45 kb and as such are particularly useful in situations where an operon consisting of several genes is to be cloned. (2) Non recombinants are selected against, as without insertion of foreign DNA, the recircularised non-recombinant cosmid particles are too small to be packaged efficiently into phage heads.

1.12.4 Phagemid vectors

Bacteriophage phagemids are another type of bacteriophage-plasmid hybrid. They are basically a plasmid with a λ*att* site which allows vector propagation either as a plasmid or a bacteriophage, in which form single stranded DNA can be generated for sequencing. Vectors in this category include

pEMBL8 (Dente *et al*, 1983) which has an f1 origin and as such can be rapidly switched between plasmid and phage modes of replication, and the pair of vectors pss24, pss25 (Kowalski *et al*, 1985) which allows copies of both DNA strands to be generated.

1.12.5 Overview

As transfection is generally more efficient than transformation, phage derived vectors (bacteriophage, cosmid and phagemid) yield a higher number of recombinants (10^{10}-10^{11}/μg DNA) than plasmids (10^{7}-10^{8}/μg DNA) (Old and Primrose, 1985). Plaques can be screened at higher densities than colonies which permits the rapid screening of large gene libraries. This is particularly useful in the case of eukaryotic libraries which may contain a large number of recombinants (Young and Davis, 1983). However, as prokaryotic libraries tend to be smaller than eukaryotic libraries (Boulnois, 1987) the advantage of high density screening is not marked. Large DNA fragments (up to 35 kb) can be cloned in cosmid or phagemid vectors which reduces primary screening of the library for identification of a positive recombinant. However, this initial advantage is minimized if extensive subcloning of the recombinant is necessary to identify and isolate the required gene within the cloned fragment. Although, in comparison, only relatively small DNA fragments can be cloned in plasmid vectors (maximum approximately 10 kb), as bacterial genomes are not large, libraries contain a level of recombinants which can be screened relatively easily. Therefore for bacterial genomic library construction, no single vector type (plasmid, cosmid or bacteriophage) appears to have a distinct advantage over any other.

The stages following library construction: maintenance, amplification and screening can be accomplished in (1) the same host strain from which the DNA originated, (2) a different host, or (3) a combination of the two.

1.13 Library maintenance and amplification in the host

Following library construction, maintenance and screening in the same host from which the DNA originated is advantageous for the following reasons: cloned fragments will be maintained in the cell, transcription/translation will be permitted and protein expressed from recombinant vectors will be stable (see Section 1.14).

The capability for transfer exists in *S. putrefaciens* based on the findings that it is possible to transfer plasmids into *S. putrefaciens* by conjugation (Gibson, 1981) and the presence of a small (< 10 kb) indigenous plasmid (Campbell, 1986). However, as yet, no cloning vectors have been constructed specifically for use in *S. putrefaciens*. The options available therefore were to use a vector derived from another species or to construct a vector from the naturally occurring *S. putrefaciens* plasmid.

A great variety of *E. coli* plasmid vectors are derived from transformable vectors such as pBR322. This plasmid has a relatively narrow host range (Sherratt, 1982) and cannot be maintained in bacteria too diverse from *E. coli*. However, the ability of *S. putrefaciens* to maintain these vectors would be advantageous, potentially increasing the selection of available cloning vectors.

Broad host range vectors like the transformable vector pGV1106 (Leemans *et al*, 1982) derived from RP4, and conjugative vector pRK290 (Ditta *et al*, 1980) derived from the incompatibility IncD plasmid group (Barth and Grinter, 1974) can be stably maintained in Gram negative bacteria such as *Vibrio* and *Pseudomonas* where *E. coli* vectors are unstable, allowing complementation studies, DNA transfer between strains, Tn5 mutagenesis and site-specific mutation of selectable markers.

Alternatively, it may be possible to construct a vector specifically for *S. putrefaciens* derived from the small indigenous plasmid. This would require extensive manipulation to insert a selective characteristic and unique restriction

sites for cloning, and to ensure the plasmid origin was maintained for stable plasmid maintenance.

1.14 Library maintenance and amplification in bacteria other than the host

There are occasions when it is advantageous to maintain a library in a host other than the one from which the cloned DNA originates. For example, eukaryotic libraries tend to be maintained and screened in *E. coli*, as this host has been extensively characterised and appropriate screening techniques are well developed (see Section 1.15). This technique can be extended to bacterial libraries when, as with *S. putrefaciens*, limited genetic characterisation of the host strain is available.

Maintenance and amplification of a library of DNA foreign to the host introduces potential problems, including host restriction of cloned DNA, instability and proteolysis of foreign protein, or non-promoter recognition by the host, inhibiting transcription/translation. These problems can be minimised by careful selection of host and vector. There are available *E. coli* strains which do not restrict foreign DNA (Young and Davis, 1984) and methylate it on subsequent replication cycles allowing stable maintenance (and amplification) of the library. Stability of expressed foreign protein can be increased in a host *E. coli* strain carrying a *lon* mutation (Mount, 1980). This is particularly important if screening requires substantial quantities of foreign protein (see Section 1.15.2). Often, cloned DNA cannot be expressed in a foreign host. This can be due to a number of factors including the failure of transcription/translation where the host RNA polymerase does not recognise a promoter sequence in the foreign DNA (Shine and Dalgarno, 1975). This problem can be circumvented by probing the library with an homologous nucleic acid probe (see Section 1.15.1). Alternatively, expression vectors may be used where expression of the cloned DNA is controlled by a functional host promoter located in the vector.

1.14.1 Expression vectors

Directed expression of foreign DNA is achieved by ligation of the DNA fragment into a unique restriction site located in a vector encoded gene, which often confers a phenotypic selection on the host, and is controlled by its own or another promoter capable of being recognised by the host RNA polymerase.

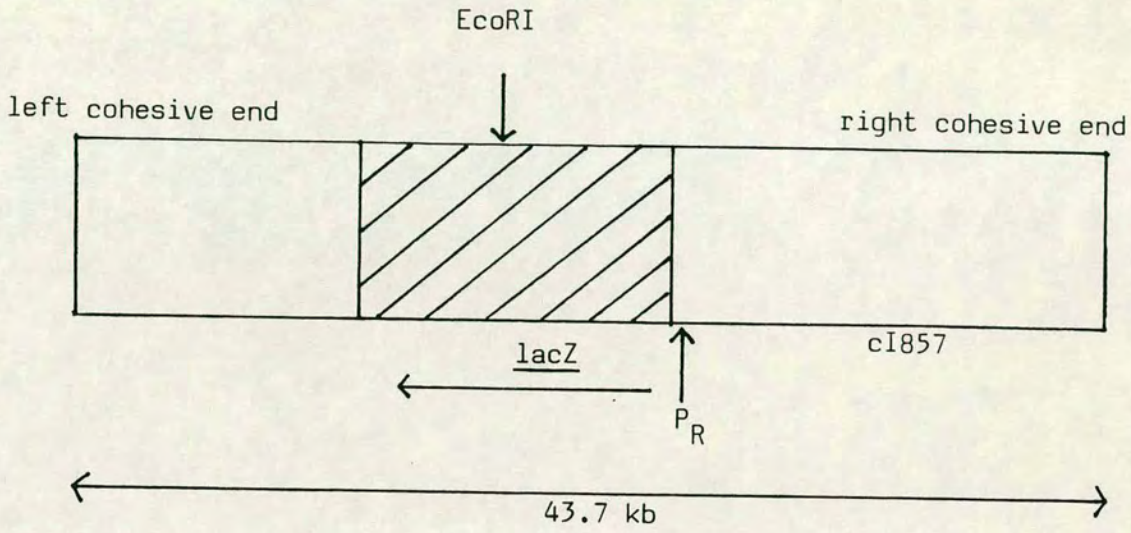
One of the first group of plasmid expression vectors described were the pUC plasmid vectors (Viera and Messing, 1982; Messing, 1983; Norrander *et al*, 1983) which contain a multiple cloning site in the α region of the *E. coli lacZ* gene (Langley *et al*, 1975). The *lac* promoter/operator region is controlled by the I^{qtss} repressor product (Muller-Hill *et al*, 1968) where normally the *qtss* mutation results in overproduction of repressor, inhibiting expression of *lacZ*. The pUC plasmids feature an elevated copy number which titrates out the *qtss* repressor, giving constitutive expression of the *lacZ* gene. As a consequence, unregulated expression of foreign DNA occurs and in some cases can be detrimental to the host resulting in cell death. This led to the construction of a number of expression vectors where tight regulation of expression combined with a strong promoter resulted in maximal expression of gene product with mimimal inconvenience to the host. Examples of these vectors discussed here are λgt11 (Young and Davis, 1983) (Figure 1.9) and the pHG group of vectors (Stewart *et al*, 1986a,b) (Figure 1.10). An extensive list of expression vectors can be found in Pouwels *et al*, 1985.

1.14.2 Regulated expression of cloned DNA

1.14.2.1 λgt11

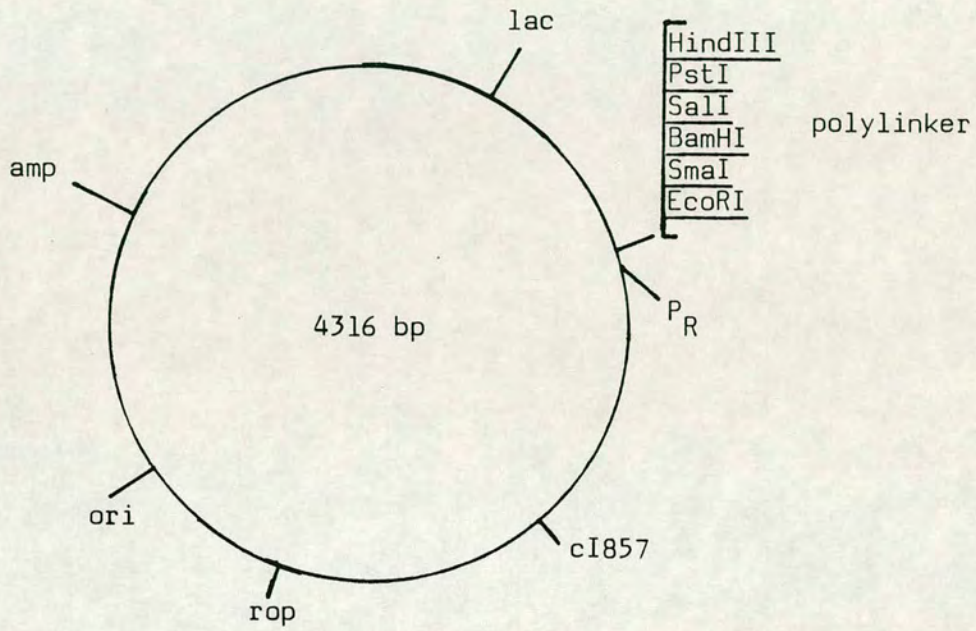
The bacteriophage fusion vector λgt11 was constructed by Young and Davies (1983), and has been used in the construction of cDNA eukaryotic libraries (Young and Davies, 1983; Kemp *et al*, 1983; Nishikimi *et al*, 1987; Kahn *et al*, 1987). λgt11 contains a unique *Eco*R1 cloning site in the *lacZ* gene

Figure 1.9 Bacteriophage λgt11



Some features of the bacteriophage expression vector λgt11. Note the unique *EcoRI* cloning site located in the *lacZ*. Expression of the *lacZ* gene is controlled by the λP_R promoter which in turn is regulated by the temperature sensitive cI857 promoter. Transcriptional orientation of *lacZ* is given by the arrow (←).

Figure 1.10 Plasmid pHG276



The main features to note are (1) the powerful λP_R promoter directing expression from the lac α gene, (2) the thermolabile cI857 repressor controlling expression of DNA from the λP_R promoter and (3) the *rop* gene to control plasmid copy number.

allowing insertion of DNA fragments up to 8.2 kb. Expression of the *l*acZ gene is controlled by the thermolabile repressor cI857 active at 30^{o}C but not at 42^{o}C. Recombinants are selected at 42^{o}C, by their *lac*$^{-}$ phenotype on agar containing IPTG, an artificial inducer of the *lac* operon, and X-gal, a colour indicator which gives white colonies or plaques when the *lac* operon is non-functional.

1.14.2.2 pHG vectors

The pHG group of plasmid vectors were derived from the elevated copy number vector pUC8 (Stewart *et al*, 1986a,b). During their construction, the pUC multiple cloning site in the α portion of *lacZ* was retained, and copy number control regained by the insertion of the *rom* gene (Twigg and Sherratt, 1980). To combine tight regulation of recombinant protein with strong expression in pHG276 the *lac* promoter was replaced by the λP_R promoter (Figure 1.10). Expression from this promoter is regulated by the thermolabile lambda repressor gene product cI857 (Grossin *et al*, 1983) active at 30^{o}C but inactive at 42^{o}C when expression of cloned DNA is permitted. Levels of foreign protein synthesis from such systems can exceed 25% of total cell protein (Zabeau and Stanley, 1982).

Although the *lac* operon has been extensively employed in expression vector systems, expression vectors have been constructed with cloning sites in operons such as *trp* where gene expression is regulated by the availability of tryptophan in the growth medium (Yanofsky *et al*, 1981). The operon is activated by transferring the plasmids to a host lacking the *trp* aporepressor gene *trpR* or by inducing starvation conditions. This type of expression vector would be unsuitable if the fusion protein had a high tryptophan content although mild starvation conditions induced by the addition of a tryptophan analogue such as indolyl 3 acrylic acid are sufficient to activate the *trp* operon without significantly affecting the fusion protein.

1.14.2.3. Advanced plasmid expression vectors

These recently discussed expression vectors, although able to regulate expression of cloned DNA, can express it in only one reading frame. Families of expression vectors such as pEX1-3 (Stanley and Luzio, 1984) and pUR (Ruther and Muller-Hill, 1983) have been developed which direct expression of cloned DNA in each of all three translational reading frames. The main advantage of this is that it maximises the chance of an expression product where the protein can adopt the correct conformation (particularly important if the library is screened by a technique depending on structural fidelity of the expressed product to native protein (Section 1.15.2).

1.14.2.4 Broad host range vectors

Broad host range expression vectors are available, based on RP4 plasmids, which allow the high expression of protein in Gram negative bacteria such as *Pseudomonas* and *Vibrio*. Plasmids with broad host range replication origins such as pPlGN1 (Leemans, 1987) allow the regulatable expression of cloned genes in a variety of Gram negative bacteria, being conjugally mobilised into a wide range of Gram negative hosts by IncP1 plasmids like pRK2073 (Leong *et al*, 1982). The advantage of this technique is that cloned DNA can be expressed at high levels in the original host bacterium.

1.15 Library screening

Commonly used methods to screen a population of recombinants for a desired gene are nucleic acid hybridisation, immunochemical detection and genetic complementation.

1.15.1 Nucleic acid hybridisation

Recombinant identification by nucleic acid hybridisation involves the use of radioactively labelled DNA (Lawn *et al*, 1978) or mRNA (Grunstein and

Hogness, 1975; Hanahan and Meselson, 1980) which is complementary to a sequence of DNA in the desired gene. This procedure is particularly useful if the cloned DNA cannot be expressed by the host. DNA or RNA probes are derived from a short amino acid sequence of the protein encoded by the gene which is to be cloned. The main disadvantage of this method stems from the redundancy of the genetic code where more than one codon can transcribe a single amino acid. To a certain extent this can be overcome by using an amino acid sequence with residues (ie tryptophan) which are transcribed by a unique codon. Additional complexity stems from bias in codon usage from gene to gene (Gouy and Gautier, 1982; Grosjean and Fiers, 1982). Furthermore, some organisms may use genetic codes different from the universal code (Fox, 1987). The present status of the genetic code has been substantiated by the data obtained from comparing DNA and protein sequence increasing the possibility of predicting the amino acid sequence for a given DNA sequence.

1.15.2 Immunochemical screening

Immunochemical methods for the detection of protein have been developed by Ehrlich *et al* (1978), and Broome and Gilbert (1979), involving incubation of recombinant colonies with antibody raised to a specific protein following which bound antibody is detected by labelled anti-IgG or protein A. The label may be enzymic, such as horse-radish peroxidase (de Wet *et al*, 1984) or alkaline phosphatase (Mierendorf *et al*, 1987) or may be radioactive I^{125} (Helfman *et al*, 1983).

This particular screening method is dependent on (a) the ability of the host cell/vector to express protein, (b) the stability of the protein within the cell, and (c) the affinity of specific antibody to the protein. Under ideal conditions, desired recombinants can be selected easily, eg a cDNA clone for human cytochrome c_1 was isolated with specific antibody from a near confluent plaque library of λgt11 (Nishikimi *et al*, 1987).

The increased use of specific antibody in immunological procedures has corresponded with detailed study on the interaction of protein antigen with the host immune response. Recent studies on myoglobin and lysozyme showed that the immunogenicity of a protein was restricted mainly to discontinuous sequences of amino acids located in surface regions (Todd *et al*, 1982; Barlow *et al*, 1986) with sequential determinants also appearing to play a part in antibody generation.

The host immune response to a protein antigen can be enhanced by the use of adjuvants on which the protein is concentrated prior to injection, leading to a high level of specific antibody in the host. The use of an adjuvant results in the localisation of the antigen, which, in conjunction with slow release of antigen from the adjuvant results in an increased immune response by reducing degradation by host proteolysis and increasing the persistence of the protein. The slow release of small amounts of the antigen is important as large doses of the protein may be toxic to the animal, or result in immunotolerance. Commonly used adjuvants are Freund's complete and Freund's incomplete adjuvants. They consist of an emulsion of mineral oil and water. Freund's complete adjuvant contains heat killed *Mycobacterium tuberculosis* which causes a heightened inflammatory response thought to be directed against the peptidoglycolipid in the cell wall.

1.15.3 Genetic complementation

This involves the restoration of a particular cell function attributable to a protein encoded by a recombinant vector clone. Ideally to clone a specific gene by this strategy, a stable host mutation in the gene is required which results in complete loss of protein activity.

Mutants can be generated by a variety of methods. Spontaneous auxotrophic mutants such as tryptophan can be readily selected on minimal agar due to mismatching of a base pair during DNA replication (Mandelstam, 1982).

Chemical mutagens are not particularly suitable for generating single mutations, giving multiple mutations randomly situated on the genome. However, nitrosoguanidine mutagenesis giving multiple closely linked mutations in replicating cells, with mutations occurring preferentially at the replication fork, has been used to assist gene cloning, including the *E. coli* fumarate reductase which was cloned by genetic complementation following nitrosoguanidine mutagenesis (Lambden and Guest, 1976).

Mutagenesis of the bacterial chromosome can also be achieved by transposable elements (Boulnois *et al*, 1985). However, natural transposons have some drawbacks, notably the mediation of additional transposition events causing genetic rearrangements such as inversion or deletions (Kleckner, 1981). Transcription may also initiate within the transposon and extend into the adjacent DNA, complicating analysis of the mutant phenotype. As a consequence of these drawbacks, engineered transposons were constructed to increase stability. This was achieved by (1) placing the tranposase outside the transposon or (2) by internal deletions thereby abolishing transposon-induced rearrangements of adjacent DNA (Way *et al*, 1984). Such an engineered transposon Omegon Km (based on IS1), has been designed specificially to allow generation of single, stable mutations in a broad range of Gram negative bacteria (Fellay, 1989). A high frequency of transposition with this transposon has been observed in *Pseudomonas putida* and lower (although still reasonable) transpositional frequencies observed with other Gram negative soil and water bacteria such as *Rhizobium leguminosarum* and *Paracoccus denitrificans*.

1.16 Summary and final perspective

Preliminary biochemical analysis of flavocytochrome c indicated that it is unique, showing no particular structural or functional similarity to other c-type flavocytochromes nor any structural similarity to the flavoprotein fumarate reductases of *E. coli* or *V. succinogenes*, although itself appeared to have a similar

function. Cloning flavocytochrome c from *S. putrefaciens* would facilitate analysis at the molecular level. The overview on the strategies available to clone flavocytochrome c showed that despite the limited knowledge of the genetics of *S. putrefaciens* advances in molecular genetics in other Gram negative bacteria provide many alternative strategies for cloning and screening flavocytochrome c.

CHAPTER 2

MATERIALS AND METHODS

2.1. Strains and plasmids.

The bacterial strains used in this study are listed in Table 2.1. Plasmids used in this study are listed in Table 2.2 and illustrated in Figures 2.1 to 2.4. Recombinant plasmids generated during this study are described in the relevant section.

2.2. Growth and maintenance of strains.

2.2.1. Growth of bacteria.

Cultures of *S. putrefaciens* were grown at 20°C and those of *E. coli* at 28°C, 30°C or 37°C as stated in the text.

2.2.2. Maintenance of Strains.

A working culture of each organism was maintained on selective agar at 4°C and subcultured every two months to check viability and purity. Permanent stock cultures were stored at -80°C in 20% glycerol.

2.3. Media.

All media were sterilised by autoclaving at 15psi for 15 min.

2.3.1. Media for *S. putrefaciens.*

Nana

Oxoid nutrient agar containing 1% NaCl.

TABLE 2.1 Bacterial Strains.

S. putrefaciens

Shewanella putrefaciens NCMB400

E. coli:

NM547 *ara B-7* δ (*lac-pro*) *rpsL* dam−

MM294 *supE*44 *hsdR endA*1 *pro thi*

JRG653 (WGAS or PL2024) *Gal trpA strep*^{r}

JRG780 (WGAS or PL2024) *Gal trpA strep*^{r} *frdA*11

TG1 *supE hsd* 5 *thi* (*lac-proAB*) F' |*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ* M15|

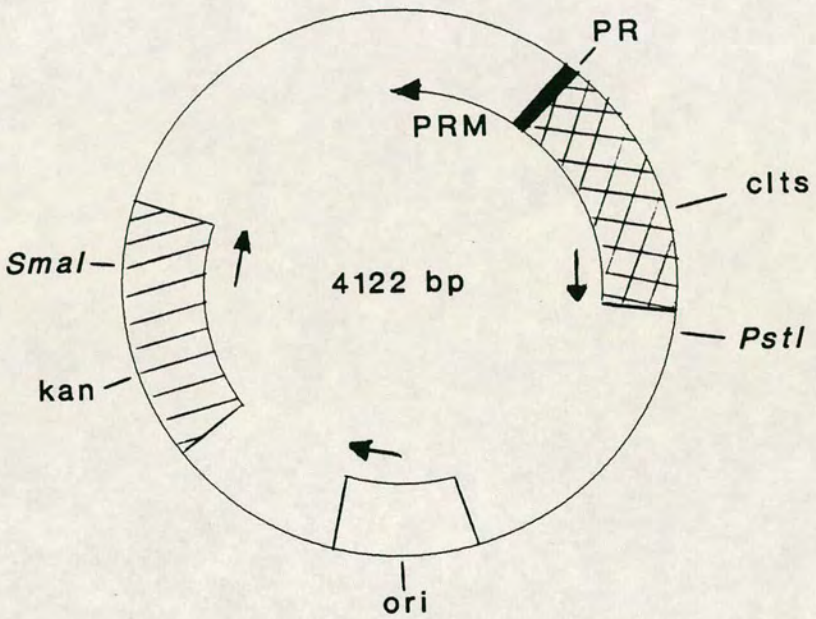
All strains were obtained from within the Department of Microbiology with the exception of JRG653 and JRG780 which were gifts from Professor John Guest, University of Sheffield.

TABLE 2.2 Plasmids.

pCI857	Remaut *et al*, (1983).
pEX1,2,3	Stanley and Luzio, (1984).
ptZ18R/ptZ19R	Rokeach *et al*, (1988).
pUN121	Nilson *et al*, (1983).

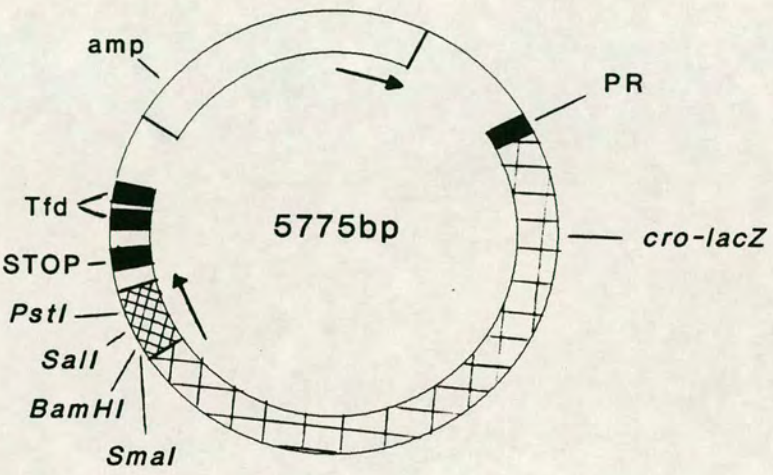
The plasmids listed above are presented in Figures 2.1.-2.4.

Figure 2.1. pcI857



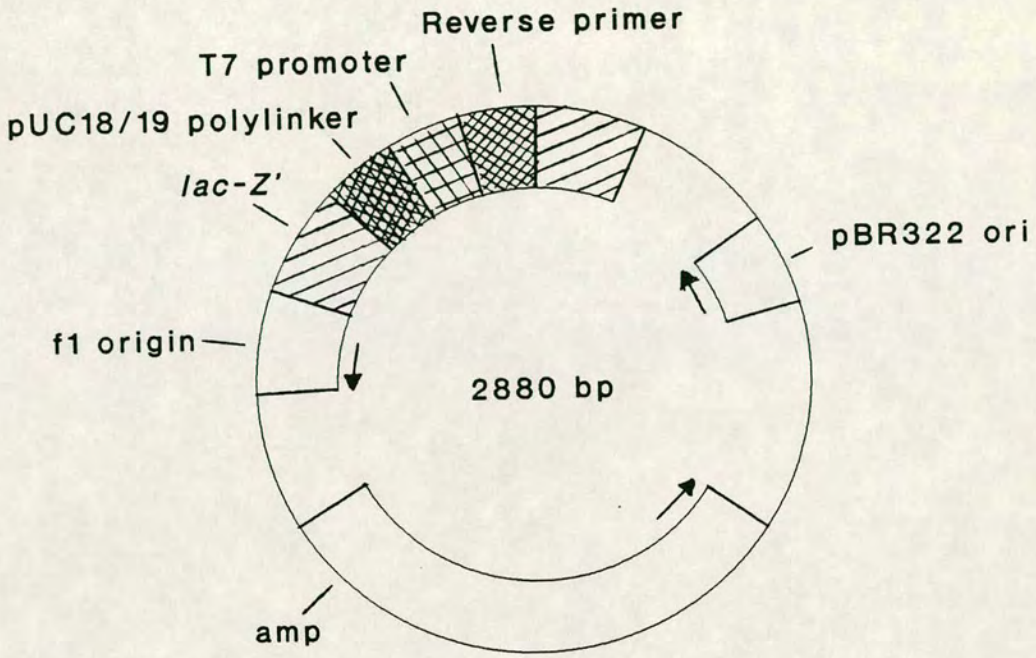
The plasmid cI857 encodes the temperature sensitive λ P_R repressor. At 30°C this repressor is active and will prevent expression of any gene controlled by the P_R promoter. A temperature shift to 42°C inactivates the repressor thus permitting expression from the P_R promoter.

Figure 2.2. pEX vectors: pEX3



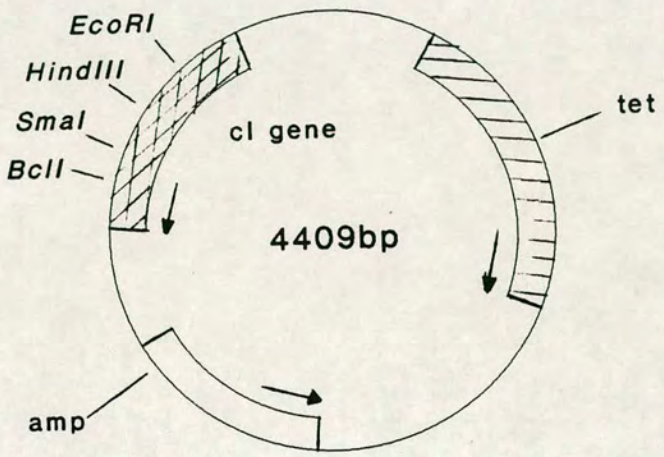
pEX1, 2, and 3 are bacterial expression vectors derived from the *cro-lacZ* from pCL19, terminator fragments from phage fd and a STOP oligonucleotide with stop codons in all three translational reading frames from pLK91, and the polylinker from pUC8. pEX1 and pEX3 were created from *Eco*RI digested pEX2 by S1 nuclease and Klenow treatment respectively, followed by screening for plasmid lacking the *Eco*R1 site. Cloned DNA is expressed at 42°C from the λ P_R promoter (controlled by the cI857 repressor), as a *cro-lacZ* fusion protein.

Figure 2.3. ptZ18R/ptZ19R



These phagemid vectors can be used for both cloning and sequencing reactions. They contain both pBR322 and f1 origins of replication allowing the generation of both ds and ss DNA respectively :production of ss DNA additionally requires the helper phage M13K07. The pUC18/pUC19 polylinker located within the *lacZ* gene of ptZ18R and ptZ19R respectively, allows cloning and identification of recombinants on X-gal plates.

Figure 2.4. pUN121



This direct selection vector was constructed with the cI gene from pTR262, and the ampicillin and tetracycline genes from pBR322 and pBR328. Inactivation of the tetracycline repressor (cI) by cloning into any of the restriction sites within this gene, confers tetracycline resistance to the host. This directly selects against non-recombinants which are tetracycline sensitive.

Wood and Baird

per litre	
NaCl	20 g
K_2HPO_4	1 g
Peptone	5 g
Yeast extract	2 g
$MgSO_4.7H_2O$	1 g

2.3.2. Media for *E. coli.*

Luria Broth

per litre	
Yeast extract	5 g
NaCl	5 g
Tryptone	10 g

Glycerol Minimal Medium

per litre	
K_2HPO_4	10.49 g
KH_2PO_4	5.44 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.05 g
$MnSO_4.4H_2O$	5 0 mg
$FeSO_4.7H_2O$	0.125 mg
$CaCl_2$	0.5 mg
Casamino acids (Difco vitamin-free)	0.5 mg

Glycerol (pH 7.2) was added to 0.04 M. L-tryptophan was added to cooled media according to Table 2.3.

Glycerol-Fumarate Minimal Media

This was essentially the same as glycerol minimal media with sodium fumarate (pH 7.2) added to 0.04 M.

Glycerol Minimal Media lacking tryptophan

This was glycerol minimal media prepared without casamino acids, and each amino acid added separately according to Table 2.3. with the exception of tryptophan.

Plates of the above media were made by the addition of 1.8% agar (Oxoid No. 3) prior to autoclaving.

2.3.3. Antibiotics.

Antibiotics were added to cooled media following autoclaving as in Table 2.4.

2.4. Chemicals and reagents.

2.4.1. Chemicals.

All chemicals used were obtained from BDH or Sigma.

2.4.2. Enzymes.

All restriction enzymes, T4 ligase, Calf intestinal alkaline phosphatase, and appropriate incubation buffers were obtained from Gibco BRL. Pancreatic

TABLE 2.3 AMINO ACIDS.

	Stock solution (%)	Plate concentration (mM)
Alanine	0.84	0.47
Arginine	2.53	0.6
Asparagine*	0.84	0.32
Aspartic acid*	1.0	0.3
Cysteine*	0.73	0.3
Glutamic acid*	18.7	5.0
Glutamine	14.6	5.0
Glycine	0.2	0.13
Histidine	0.31	0.1
Isoleucine	0.79	0.3
Leucine	0.79	0.3
Lysine	1.1	0.3
Methionine	0.9	0.3
Phenylalanine	0.99	0.3
Proline	4.6	2.0
Serine	8.4	4.0
Threonine	0.71	0.3
Tryptophan*	0.41	0.1
Tyrosine	0.36	0.1
Valine	0.7	0.7

All amino acids were added to the media before autoclaving with the exception of those marked * which were filter sterilised and added to the cooled media after autoclaving. The addition of 5 ml stock solution per litre of media resulted in the appropriate plate concentration (Davis *et al.*, 1980).

TABLE 2.4. Antibiotic concentrations for *E. coli*.

Antibiotic	concentration
Ampicillin	100
Kanamycin	50
Tetracycline	14

Values are expressed as final concentration (μg ml^{-1}). Ampicillin and kanamycin stock solutions were 25 mg ml^{-1} in aqueous solution and sterilised by filtration. Tetracycline stock solution was 12.5 mg ml^{-1} in ethanol:water (50% v/v). All were stored at -20^{o}C.

ribonuclease A was obtained from Sigma. Removal of DNase activity was achieved by dissolving the RNase A at 100 μg ml^{-1} in 10 mM Tris-HCl (pH 7.5) 15 mM NaCl and heating to 100°C for 15 min.

2.4.3. Antibody.

Sheep/Goat anti-IgG Horse radish peroxidase was obtained from the Scottish Antibody Production Unit (SAPU).

2.5. Preparation of periplasm from *S. putrefaciens.*

Periplasm from *S. putrefaciens* was prepared by a modification (Easter 1982) of the osmotic shock/lysis procedure of Birdsell and Cota Robles (1967). *S. putrefaciens* NCMB400 was grown microaerobically in broth (pH 7.2) containing 10 mM fumarate and 20 mM lactate to reach an A_{660} of 0.2-0.3. The cells were harvested by centrifugation at 4°C for 15 min at 10 000g, then washed in buffer (100 mM K_2HPO_4 pH 7.2; 100 mM NaCl; 1 mM $MgSO_4.7H_20$). The pellet was resuspended in 100 mM Tris/HCl (pH 8.0) containing sucrose (0.5 M) and incubated statically at room temperature for 10 min. Lysozyme (1 mg ml^{-1}) was added to a final concentration of 30 μg ml^{-1} and incubation continued for a further 10 min. The cells were shocked on addition of an equal volume of 10 mM Tris/HCl (pH 8.0) with continuous stirring , followed 10 min later by EDTA to a final concentration of 1 mM. Sphaeroplast formation was monitored by phase contrast microscopy throughout these procedures, usually yielding 90-100% sphaeroplasts within 15 min of EDTA addition. The periplasm was collected by centrifugation of the shocked cells at 17 000g for 15 min.

2.6. Column chromatography.

Protein concentrations, loading and elution rates appropriate for each matrix type were optimised according to the manufacturers' guidelines.

2.6.1. Ion exchange.

Ion exchange chromatography was carried out using the cation exchanger DEAE sepharose CL-6B in a column of bed dimensions 2.6 x 14.5 cm. The column was equilibrated with 10 mM Tris-HCl pH 8.4 (T-buffer) before loading of samples then washed with 3 column volumes of T-buffer to remove unbound material. The column was developed with an increasing linear gradient of NaCl (0-0.5 M, 800 ml) in T-buffer at 8 ml h^{-1}.

2.6.2. Hydroxyapatite.

The matrix (bed dimensions 2.6 x 12.5 cm) was equilibrated in T-buffer and the protein sample loaded and washed as for ion exchange chromatography before developing with an increasing linear gradient of K_2HPO_4 pH 8.4 (0-0.5 M, 500 ml) in T-buffer at 8 ml h^{-1}.

2.6.3. Phenyl sepharose.

Phenyl sepharose (bed dimensions 2.6 x 11.5 cm) was equilibrated in T-buffer containing 2 M NaCl prior to loading of the sample which was prepared in the same buffer. The column was washed with 3 column volumes of equilibration buffer before development with a decreasing linear gradient of NaCl (2-0 M, 500 ml) in T-buffer at 12 ml h^{-1}.

2.6.4. Gel Filtration.

A Sepharose 4B column of bed dimensions 1 x 88 cm was equilibrated with T-buffer before loading of the sample which was eluted with 0.2 M NaCl in T-buffer at 12 ml h^{-1}.

2.6.5. Sample collection and preparation.

Fractions (4 ml) were collected in acid washed tubes and the A_{280} and A_{410} of each fraction measured to determine respective protein and haem content. Elution profiles for protein and haem were then obtained by plotting A_{280} and A_{410} values against fraction number. Flavocytochrome containing peaks were pooled, concentrated by dialysis against solid polyethylene glycol at 4°C, then equilibrated by dialysis against 1000 volumes of T-buffer at 4°C.

2.7. Ammonium sulphate precipitation of periplasm.

Ammonium sulphate precipitation of periplasm (section 2.5.) was achieved by slow addition of solid ammonium sulphate (specially low in heavy metals for enzyme work). Periplasmic protein was precipitated on incubation at 4°C for 2 h with the appropriate amount of ammonium sulphate related to the volume of periplasm and the present and required degree of ammonium sulphate saturation (Green and Hughs, 1955). Precipitated protein was removed by centrifugation at 30 000g for 30 min at 4°C.

2.8. Protein determination.

Protein concentrations were determined using the consolidated method of Peterson (1977) based on the Folin-Ciocalteau phenol reagent method as given by Lowry *et al* (1951). This method allows the rapid quantitative recovery of soluble and membrane proteins from interfering substances by prior

precipitation of the protein followed by estimation with the Folin-Ciocalteau phenol reagent.

To a sample of protein (10-100 μg) in a volume of 1 ml was added 100 μl sodium deoxycholate (0.15% (w/v)). The solutions were mixed and allowed to stand at room temperature (20°C) for 10 min before the addition of 100 μl Trichloroacetic acid (72% (w/v)). Precipitated protein was pelleted by centrifugation at 12 300 g for 15 min in an Eppendorf centrifuge (Microspin 12, Sorvall Instruments, Dupont). The supernatant was discarded leaving the pellet of precipitated protein for assay. To the precipitated protein was added sterile distilled water (1 ml) and Reagent A (1 ml) which consisted of equal proportions of copper tartrate carbonate (copper sulphate (pentahydrate), 0.1%(w/v); sodium potassium tartrate, 0.02%(w/v); sodium carbonate, 10%(w/v)), sodium hydroxide (0.8 M), sodium dodecyl sulphate (10%(w/v)) and sterile distilled water. The solutions were mixed and incubated at room temperature for 10 min to allow the protein to solubilise. Reagent B (Folin-Ciocalteau phenol reagent, 16.7% aqueous solution) (0.5 ml) was added and mixed immediately. The colour was allowed to develop for 30 min before reading the absorbance at 750 nm against a reagent blank. The protein concentration of the unknown samples was determined by reference to a standard curve of 10-100 μg protein (BSA) which was produced for each assay.

2.9. Raising antibody to purified flavocytochrome c.

2.9.1. Treatment of sheep.

Purified flavocytochrome (2 ml of a 0.5 mg ml^{-1} solution in phosphate buffered saline (Oxoid)) was filter sterilised and added to 2 ml Freund's complete adjuvant then emulsified by stirring at (20 000 rpm) for 1 min in an

Ultra-Tumix homogeniser. The protein-adjuvant emulsion was injected into sheep 762N at 4 sites intramuscularly. The sheep was boosted 2 weeks later with 0.5 mg flavocytochrome in Freund's incomplete adjuvant prepared as before. A sample of preimmune serum was taken prior to the first injection, with bleeding carried out after 2 weeks and then at weekly intervals (removing 50-100 ml of blood each time) until a good antibody response to the flavocytochrome was obtained.

2.9.2. Preparation of antiserum from sheep blood.

Sachets for the collection of human blood were obtained from the Scottish National Blood Transfusion Service. The contents (anhydrous citric acid, 0.3 g, sodium citrate-dihydrate, 2.63 g, sodium phosphate, 0.22 g, glucose monohydrate, 3.83 g, pH 5.7 at 20°C) were dissolved in 63 ml sterile water then added to 450 ml sheep blood. The suspension was centrifuged (4000 g for 30 min at 20°C to remove red blood cells then the plasma was clarified by centrifugation (10 000 g for 15 min at 20°C). Clotting of the plasma was achieved within 1 h at 37°C following addition of $CaCl_2$ (400 mM) in the ratio 1:20 (1 vol. $CaCl_2$ to 20 vol. plasma). The plasma clot was removed leaving the sheep antiserum.

2.9.3. Precipitation of IgG from antiserum.

Immunoglobulin G was prepared from sheep antiserum by the caprylic acid fractionation method of Steinbuch and Audran, (1969). Antiserum was placed in a glass beaker with 2 vol. of 0.06 M sodium acetate pH 4.0 and stirred, avoiding frothing, using a magnetic stirrer. N-octanoic acid (7.5 ml per 100 ml of original serum volume) was added dropwise to the antiserum/buffer solution using a syringe with a 19 gauge needle. The solution was stirred vigorously

throughout addition of the octanoic acid and stirring continued for a further 30 min after complete addition before collecting the precipitate by centrifugation at 2000 g for 30 min at 20°C. The supernatant was filtered through Whatman grade 4 filter paper to remove fines prior to removal of octanoic acid by dialysis against 0.154 M NaCl. The IgG solution was stored in small aliquots at -20°C.

2.10. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli (1970) in the presence or absence of sodium dodecyl sulphate (SDS). Resolving linear gradient gels of 7.5% - 15% were used to gain effective separation of samples containing a variety of proteins with the purified flavocytochrome electrophoresed on 10% resolving gels. Stock acrylamide solution was prepared from acrylamide 29.2% (w/v), NN'-Methylenebisacrylamide, 0.8% (w/v) (Electran grade) in aqueous solution and stored at 4°C in the dark. Resolving gels and stacking gels (4.5%) were prepared with the solutions given in Table 2.5. Immediately after pouring, the resolving gel was overlaid with water saturated isobutanol to ensure a level top during polymerisation (30-40 min). The stacking gel solution was added to the polymerised resolving gel. Samples for electrophoresis (10-50 μl) were prepared 1:1 in sample buffer (10 mM Tris-HCl pH 6.8 containing glycerol, 10% (v/v); SDS, 2% (w/v); mercaptoethanol; and bromophenol blue, 0.005% (w/v) for SDS gels or glycerol, 10% bromophenol blue, 0.005% (w/v) for native gels. Protein samples for silver staining were adjusted to give an equivalent protein loading of 10 μg in each sample ; cytochrome samples for haem staining were adjusted to an A_{410} of 0.25 in 30 μl. Samples were electrophoresed in electrode buffer (25 mM Tris, 200 mM glycine, +/- 0.1% SDS) at 15 mA constant current

TABLE 2.5 Solutions for PAGE.

	% Acrylamide			
	15%	**10%**	**7.5%**	**4.5%**
1.5 M Tris pH8.8 (+/- 0.2% SDS)	3.75	3.75	3.75	-
0.5 M Tris pH6.8 (+/- 0.2% SDS)	-	-	-	3.75
Acrylamide stock	7.5	5	3.75	2.25
d.$H_2$0	2.1	6.25	7.5	9
Glycerol	1.5	-	-	-

All volumes are expressed in ml.

The polymerising agents ammonium persulphate (10% fresh solution) and Temed were added to a final concentration of 0.02% (v/v) immediately before pouring the gels.

for 1.5 h followed by 25 mA constant current until the tracking dye had reached the base of the gel.

2.11. Staining of acrylamide gels.

2.11.1. Silver stain.

The Bio-rad silver stain was used for the detection of low (< 10 μg) amounts of protein. Oxidising, silver and developing solutions were prepared immediately before use in distilled deionised water (ddH_2O). Gels were immersed in Fixative A (40% methanol, 10% glacial acetic acid (v/v)) for at least 30 min then washed for 15 min x 2 in Fixative B (10% ethanol, 5% glacial acetic acid (v/v)). The gels were immersed in oxidiser for 5 min (0.2 g $K_2Cr_2O_7$ in 200 ml ddH_2O containing 40 μl concentrated HNO_3) then washed for 5 min x 2 in ddH_2O. After staining for 20 min in the silver reagent (0.4 g $AgNO_3$ in 200 ml ddH_2O) the gels were washed for 1 min in ddH_2O then rinsed for 30 s x 2 in developer (Na_2CO_3, 18 g in 600 ml ddH_2O containing 0.3 ml formaldehyde). Gels were then placed in developer and the appearance of bands monitored visually. Development of bands was arrested by placing the gels in 0.5% glacial acetic acid.

2.11.2. Kenacid blue.

The kenacid blue stain was used to check successful protein transfer following Western blotting. Gels were stained overnight with gentle agitation in kenacid blue (0.1%) in water:methanol:glacial acetic acid (5:5:2 by volume) then destained in frequent changes of destain solution (30% methanol (v/v) and 10% glacial acetic acid (v/v) in aqueous solution) and stored in 0.5% glacial acetic acid (aqueous solution).

2.11.3. Haem stain.

Gels were stained for cytochrome by a modification of the peroxidase method of Thomas *et al.* (1976). 3,3',5,5'-Tetramethylbenzidine (TMBZ) (40 mg) was dissolved in 39 ml methanol immediately before use then added to 70 ml 0.25 M sodium acetate pH 5.0 . Gels were equilibrated in methanol/sodium acetate pH 5.0 (3:7) for 5 min then placed in the TMBZ solution. Staining was carried out in the dark for 15 min with gentle agitation every 5 min. On addition of H_2O_2 (1 ml of a 30% v/v solution) the formation of blue cytochrome bands was observed in subdued light. Once full intensity was reached (30 min) the gels were fixed in isopropanol/0.25 M sodium acetate pH 5.0 (3:7) and stored in 5% acetic acid.

2.11.4. Zymogram.

Fumarate reductase activity of protein bands following PAGE under non-denaturing conditions was detected by the zymogram stain. Zymogram buffer (100 mM Tris pH 7.8; 100 mM K_2HPO_4; 7.5 mM $KHCO_3$; 2 mM methyl viologen) was rendered oxygen free by continuous sparging with argon throughout all stages of the staining procedure. Reduction of methyl viologen was observed by the immediate colour change of the buffer to deep blue on addition of sodium dithionite to 5 mM. The gel was immersed in the reduced buffer and allowed to absorb the dye (10-15 min). On addition of sodium fumarate (10 mM final concentration) enzyme activity was apparent as clear zones on a blue background. The gel was photographed immediately following staining as methyl viologen is oxidised rapidly in the presence of oxygen.

2.12. Western blotting.

2.12.1. Protein transfer to membrane.

The electrophoretic transfer of proteins from acrylamide gels to Nylon membranes was essentially that described by Gershoni and Palade (1983). Proteins were separated on SDS-PAGE as described previously. Nylon membrane (Amersham Hybond N) was cut to the same size as the gel. Gel, membrane, two sponges and two sheets of Whatman 3MM filter paper were soaked in transfer buffer (25 mM Tris HCl pH 8.3; 1.5 M glycine) for 5 min then fitted together as a sandwich in the plastic holders with gel and membrane next to each other enclosed on either side by a piece of filter paper and a sponge. The sandwich was placed in the transfer tank with the gel oriented towards the negative terminal and proteins were transferred for 2.5 h at 1.0 Amp in transfer buffer. The gel and membrane were removed from the sandwich : the gel stained in kenacid blue to check efficiency of transfer, and the membrane blocked overnight in 20% milk powder in TBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl) prior to developing with protein specific antibody.

2.12.2. Antibody Detection.

The blocked membrane was placed in 5% milk solution (40 ml) in TBS containing antisera (30 μl) and shaken gently at room temperature for 5 h. The membrane was washed 4 x 5 min in TBS before placing in a solution of 5% milk (40 ml) in TBS containing HRP-conjugated antisheep IgG (20 μl) and incubated as before for 2 h. Washings were repeated. The membrane was removed from the TBS and placed in developing solution (1 mM dianisidine, 10 mM imidazole pH 7.4, 0.1 ml 30% v/v H_2O_2) (to 10 ml in d.H_2O). The reaction was stopped

by transferring the membrane to distilled water. The membrane was dried and photographed then stored in the dark.

2.12.3. Dot Blotting.

Dilutions of purified flavocytochrome were spotted on to strips of dry nylon membrane and allowed to dry. The strips were blocked and developed according to section 2.12.1.

2.13 Enzyme assays.

2.13.1 Fumarate reductase.

Fumarate reductase activity was assayed by the anaerobic fumarate dependent reoxidation of reduced methyl viologen at 600 nm. The reaction mixture contained enzyme source (10 - 100 μl), sodium fumarate pH 6.5 (initially 10 mM then x 10 Km value) and buffer (100 mM K_2HPO_4, 100 mM NaCl, 1 mM $MgSO_4.7H_2O$; pH 7.2 containing 0.3 mM methyl viologen) to a total volume of 4 ml and was assayed in a Hellma 21N stoppered cuvette.

Enzyme source (100 μl) and buffer sparged with oxygen-free nitrogen were used to fill the cuvette and a few antibumping granules added. The teflon stopper (drilled to take a 10 - 100 μl syringe needle) was carefully inserted to exclude all air and sodium dithionite (a fresh solution of 25 mM in 10 mM NaOH) added by syringe to give an A_{600} reading of 1.0 after mixing by inversion. The endogenous enzyme activity was measured for 2 min before the reaction started by addition of fumarate (100 μl). The enzyme activity was determined from the rate of decolourisation at 600 nm assuming an extinction coefficient of 13 μmol cm^{-1} ml^{-1} for methyl viologen. (Thorneley, 1974).

2.13.2. Succinate Dehydrogenase.

Succinate dehydrogenase activity was measured by the reduction of DCPIP at 600 nm (Ells, 1959). The reaction was assayed in a 1 ml Hellma cuvette containing (final concentration) 79 mM Tris-HCl pH 7.4, 10 mM sodium cyanide (a fresh solution) pH 7.0, 0.1 mM DCPIP, 1.0 mM PMS, sodium succinate pH 7.0 (0.1-1.5 mM in 100 μl) and enzyme source (100 μl) in a total volume of 1 ml. Endogenous enzyme activity was measured for 2 min before the reaction was started by addition of succinate. The enzyme activity was measured at 600nm assuming an extinction coefficient of 21 μmol cm^{-1} ml^{-1} for DCPIP (Lester and DeMoss, 1971).

2.14. Antibody precipitation of flavocytochrome c.

The ability of antibody raised against the flavocytochrome to precipitate fumarate reductase activity was tested by preincubation of purified flavocytochrome and fumarate grown periplasm with specific IgG before assaying for fumarate reductase activity (section 2.13.1). Samples were incubated on ice for 10 min prior to assay.

2.15. Isolation of plasmid DNA.

Plasmid DNA was isolated using the alkaline lysis method essentially as described by Birnboim and Doily (1979).

2.15.1. Small scale preparation.

This method was used to yield approximately 5 μg plasmid DNA for restriction analysis. A single colony was grown overnight in 5 ml selective broth. Cells were harvested at room temperature for 10 min at 3000 g and the pellet

resuspended in TEG (25 mM Tris-HCl pH 8.0; 10 mM EDTA; 50 mM glucose) (200 µl) containing fresh lysozyme (10 mg ml^{-1}) then transferred to a 1.5 ml Eppendorf tube. After incubation on ice for 5 min lysis solution (0.2 M NaOH, 1% SDS) (400 µl) was added and incubation continued for a further 5 min before 3 M sodium acetate pH 5.0 (300 µl) was added to precipitate cell protein. The sample was incubated on ice for 10 min before centrifugation at 0°C for 15 min at 13 000g. Supernatant (750 µl) was removed to a clean tube to which isopropanol (450 µl) was added. The tube contents were well mixed and incubated at room temperature for 10 min. DNA was pelleted by centrifugation at room temperature for 5 min at 13 000g. The supernatant was discarded and the pellet washed x 5 in chilled (-20°C) ethanol (70%), dried under vacuum and resuspended in TE (100 µl).

2.15.2. Medium Scale preparation.

This preparation was used to yield approximately 40 µg of reasonably pure DNA for restriction analysis, cloning and transformations. A single colony was grown overnight in 40 ml selective broth. Cells were harvested at 15°C for 5 min at 10 000g then resuspended in TEG (2 ml) containing freshly added lysozyme (2 mg ml^{-1}). The cells were incubated on ice for 30 min. Lysis solution (4 ml) was added, the tube vortexed briefly, then left on ice for 5 min before the addition of 3 M sodium acetate pH 5.0 (3 ml). Incubation on ice was continued for a further 30-60 min then cell debris removed by centrifugation at 4°C for 30 min at 9000g. The supernatant was transferred to a clean tube to which cold ethanol (16 ml) was added then incubated at 0°C for 15 min before centrifugation at 0°C for 10 min at 9000g. The pellet was dissolved in 40 mM Tris pH 8.0, 1 mM EDTA, 100 mM sodium acetate, 0.1% SDS, (2 ml). Phenol: chloroform: isoamylalcohol (50:50:1) (2 ml) was added and mixed by vortexing

before centrifugation at 15°C for 15 min at 5000g. The aqueous phase was removed to a clean tube and the phenol and interphase re-extracted with buffer (2 ml). The aqueous phases were combined and extracted with chloroform (4 ml). Nucleic acid was precipitated on addition of cold ethanol (8 ml) followed by incubation at -20°C for 15 min, and collected on centrifugation at 0°C for 15 min at 9000g. The pellet was dissolved in 400 μl TE and transferred to a 1.5 ml microfuge tube to which 4 M NaCl (20ul) and cold ethanol (1 ml) were added. The tube was incubated at -20°C for 10 min then centrifuged at 0°C for 5 min at 13 000g. RNA was removed from the sample by addition of DNase-free RNase (20 μl of a 1 mg ml^{-1} solution) to the resuspended pellet (TE; 200 μl) followed by incubation at 37°C for 1 h. RNase was inactivated and removed by phenol: chloroform: isoamylalcohol (50:50:1) (400 μl) extraction in the presence of 4 M NaCl (20 μl) and sterile distilled water (200 μl). DNA was precipitated from the aqueous phase on addition of cold ethanol (800 μl) followed by incubation at -20°C for 10 min then collected on centrifugation at 0°C for 5 min at 13 000g. The pellet was dissolved in TE (200 μl).

2.15.3. Single stranded DNA preparation.

Single stranded DNA was obtained from plasmids with the f1 origin of replication (phagemids) propagated in *E. coli*. The culture was grown to reach an A_{600} of 0.5-0.8 then 2 ml was infected with M13KO7 helper phage at a multiplicity of infection of 10. Following incubation with vigorous shaking (240 rpm) for 1 h, 400 μl of the infected cells were transferred to fresh broth containing ampicillin and kanamycin (70 μg ml^{-1}) and grown overnight. The supernatant of 1.5 ml overnight culture was mixed with 0.3 ml 2.5 M NaCl, 20% PEG 6000 then spun for 5 min after incubation at room temperature for 15 min to harvest the phage. The supernatant was completely removed and the pellet

resuspended in TE (100 μl). Phage DNA was released by extraction with Tris buffered phenol followed by extraction with chloroform. Phage DNA was precipitated by sodium acetate pH 5.0 (0.1 vol) and ethanol (2 vol) at -20°C for 1 h then collected by centrifugation for 5 min at 0°C. The pellet was dried and resuspended in TE (50 μl).

2.16. DNA Manipulation Techniques.

2.16.1. Restriction endonuclease digestion.

DNA was digested in a total volume of 10 μl. Enzyme (1 unit) and x 10 enzyme buffer (1 μl) were added to DNA (approximately 1 μg) and incubated for 4 h. For restriction of increased concentrations of DNA the digestion mixture was scaled up accordingly. Restriction enzymes used in this study are listed together with their appropriate buffers in Table 2.6.

2.16.2. Ligation of DNA.

Ligation of DNA was performed in a total volume of 10 μl. Subcloning of isolated DNA fragments into another vector generally required a vector fragment ratio of 1:3 although for blunt ended ligations an increased concentration of both DNA species was used. The relative concentrations of fragment and vector used in construction of the gene libraries were optimised to yield a large number of recombinants. The final ligation mix contained DNA (200 ng) in ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG 8000) containing T4 ligase (1 unit). Ligation was carried out for 4 h at 20°C or overnight at 15°C.

TABLE 2.6 Restriction enzymes and digestion buffers

Buffer 1	Buffer 2
50 mM Tris-HCl pH 8.0	50 mMTris-HCl pH 8.0
100 mM $MgCl_2$	10 mM $MgCl_2$
100 mM NaCl	

Buffer 3	Buffer 4
50 mM Tris-HCl pH 8.0	20 mM Tris-HCl pH 7.4
10 mM $MgCl_2$	5 mM $MgCl_2$
100 mM NaCl	50 mM KCl

Buffer 6	Buffer 7
50 mM Tris-HCl pH 7.4	50 mM Tris-HCl pH 8.0
6 mM $MgCl_2$	10 mM $MgCl_2$
50 mM KCl	50 mM KCl
50 mM NaCl	

1	2	3	4	6	7
*Dra*I	*Hind*III	*Eco*RI	*Sau*3A	*Pvu*II	*Nru*I
*Cla*I	*Pst*I	*Bam*HI	*Sma*I	*Sca*I	
	*Bcl*I				
	*Bgl*I				
	*Stu*I				

The optimum incubation temperature for the above restriction enzymes was 37^{o}C with the exception of *Sma*I (30^{o}C) and *Bcl*I (50^{o}C).

2.16.3. Alkaline Phosphatase Treatment of DNA.

Vector ends were treated with alkaline phosphatase to prevent self ligation therefore increasing the chance of recombinant formation. Calf intestinal alkaline phosphatase was added to digested plasmid DNA (1 unit per μg DNA) and incubated at 37°C for 1 h. EGTA was added to 20 mM (stock 100 mM pH 8.0) and incubation continued at room temperature for 20 min. Alkaline phosphatase was removed by extraction of the DNA solution twice with Tris buffered phenol and once with chloroform and the phosphatased DNA collected by ethanol precipitation.

2.17. Electrophoresis of DNA.

2.17.1. Agarose gel electrophoresis.

Agarose gels were used for the electrophoretic separation of DNA fragments and unless otherwise stated were used at a concentration of 0.8%. Agarose was dissolved by heating in Tris acetate buffer (TAE, 40 mM Tris, 1 mM EDTA, 20 mM sodium acetate; pH adjusted to 8.2 with acetic acid) and cooled to 40°C before addition of ethidium bromide to a final concentration of 1 μg ml^{-1}. The agarose was poured into a gel cast and allowed to set then immersed in TAE before DNA samples containing tracking dye (Ficoll bromophenol blue, 1% in distilled water) were loaded. The samples were electrophoresed at 100mA until the bands of DNA had fully resolved. This was monitored by examination of the gel at 280nm using ultraviolet light.

2.17.2. Isolation of DNA fragments.

Fragments of DNA were recovered from agarose by the Geneclean II Kit (BIO 101 Inc.) which also supplied the appropriate solutions. DNA was

electrophoresed on agarose (section 2.17.1) and the required DNA fragment cut out of the gel using a sharp blade. To the fragment was added sodium iodide solution (x 3 vol. the weight of the fragment). Agarose was dissolved by heating at 50°C for 5 min followed immediately by chilling on ice. Glassmilk (5 μl) was added to the solution and incubation on ice continued for 5 min with regular mixing to ensure suspension of the particles. The glassmilk matrix was pelleted following centrifugation (5 s) then the NaI supernatant was decanted and the pellet washed x 3 with New Wash. DNA was eluted from the glassmilk by resuspension of the pellet in TE (5 μl) followed by heating at 50°C for 3 min then centrifugation (30 s). The supernatant containing the eluted DNA was transferred to a clean tube and elution of the glassmilk repeated.

2.17.3. Sequencing gels.

Urea acrylamide gels were used for the separation of nucleotides. Gels were cast as slabs of dimensions 33 x 39.5 x 0.05 cm with the combs (BRL sharkstooth) inverted 0.3 mm below the level of the smaller gel plate. Following polymerisation the combs were removed and reinserted between the gel plates to form wells with the comb teeth piercing the acrylamide. The gel was placed in electrophoresis apparatus (BRL) and prerun for 60 min in TBE at 65 volts prior to loading of samples.

2.18. DNA sequencing.

Single stranded DNA was sequenced by the dideoxy method as performed by Sanger *et al* (1977).

2.18.1. Labelling of single stranded DNA.

DNA was labelled according to the Sequenase kit who supplied all sequencing reagents (with the exception of ^{35}S d-ATP).

2.18.2. Sequencing of labelled DNA.

Labelled DNA (3 μl) was electrophoresed (section 2.17.3.) for 1.5 h, 3 h and 6 h at constant 65 volts to gain maximum sequence information. Following electrophoresis the gel was immersed in for 10 min then dried for 1 h on Whatman 3MM paper before exposing overnight at room temperature.

2.18.3. Sequence analysis

All programs used for analysis of DNA and amino acid seqence were present in the University of Wisconsin Genetics Group (UWGG) program package. Nucleotide sequence was analysed by the program FASTA then translated by the program MAP. Amino acid sequence was analysed by the Swissprot and OWL databases.

2.19. Southern blotting.

2.19.1. Transfer of DNA to membrane.

2.19.1.1. Transfer of DNA from agarose

The capillary blotting method used for transfer was essentially that described by Southern (1975). Chromosomal DNA (1 μg) from *S. putrefaciens* was suitably digested with appropriate restriction enzymes and electrophoresed on agarose (section 2.17.1.). DNA was denatured by placing the gel in 100 ml denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 x 20 min followed by neutralising in

100 ml 3 M NaCl, 0.5 M Tris-HCl pH 7.5 for 2 x 20 min. The gel was inverted on to 3MM Whatman filter paper presoaked in 20 x SSC (3 M NaCl. 0.3 M sodium citrate), the ends of which were resting in a reservoir of 20 x SSC. Hybond-N cut to the exact dimensions of the gel was placed on to the surface of the gel followed by 3 sheets of 3MM Whatman filter paper presoaked in 2 x SSC. Paper towels were stacked on top to a height of 10 cm ensuring that they did not contact the reservoir of 20 x SSC. A heavy weight was placed on top and transfer carried out overnight. The filter was allowed to air dry then wrapped in Saran wrap before fixing DNA to the filter by UV light for 4 min.

2.19.1.2. Transfer of DNA from colonies

Colonies were grown up overnight to the required size. Filters (Hybond N, Amersham) were placed on the surface of the colonies and orientation marks pricked through the filter on to the agar. The filters were placed colony side up on to a pad of filter paper soaked in denaturing solution (7 min) then removed to neutralising solution (2 x 3 min). Filters were washed briefly in x 2 SSC, air dried before wrapping in Saran wrap and fixing the DNA by placing colony side down on a UV transilluminator for 5 min.

2.19.2. Synthesis of DNA probes

DNA to be labelled (50 ng) was denatured in a volume of 32.5 µl by boiling for 5 min. The DNA was rapidly cooled on ice before addition of 10 µl OLB (see below) and 2 µl BSA (10 mg ml^{-1}) then annealed for 10 min at 37°C. The DNA was labelled on addition of (^{32}P)- dCTP (5 µl) to the DNA solution with 2 units of Klenow fragment of DNA polymerase followed by incubation at room temperature. Labelling was complete after incubation at room temperature for 2.5-5 h.

Solutions for probe synthesis

Solution O	1.25 M Tris-HCl pH8.0 , 125 mM $MgCl_2$
Solution A	1 ml solution O, 18 µl 2-mercaptoethanol, 5 µl each of dATP, dGTP, dTTP
Solution B	2 M Hepes pH 6.6
Solution C	Hexadeoxyribonucleotides in TE at 90 OD units ml^{-1}.
Nucleotide Stocks	dATP, dGTP, dTTP were at a concentration of 0.1 M in 3mM Tris-HCl pH 7.0, 0.2 mM Na_2EDTA.
OLB	Solutions A:B:C: in a ratio of 10:25:15

2.19.3. Detection of homologous DNA.

Prehybridisation / Hybridisation Solution

6 x SSC

1% SDS

5 x Denhardts (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrollidine)

Filters prepared as in section 2.19.1.2. were placed in a sealed bag with the appropriate amount of prehybridisation solution (25 ml per 100 cm^2 filter) containing 0.1% denatured calf thymus DNA (DNA was denatured by boiling for 5 min prior to use). The membrane was incubated at 65°C for 4 h then the prehybridisation solution replaced with hybridisation solution containing denatured labelled probe (section 2.19.2.). Hybridisation was at 65°C for 12 h. The filter was washed x 2 for 15 min at 65°C in x 2 SSC then for 30 min at 65°C in x 2 SSC containing 1% SDS. The filters were air dried then wrapped in Saran wrap and exposed for 2 - 5 days at -70°C.

2.20. Transformation of plasmid DNA into *E. coli.*

E. coli was transformed with plasmid DNA essentially as described by Mandel and Higa (1970).

2.20.1. Competent cells.

A culture was grown aerobically from a single colony to reach an A_{600} of 0.3. Cells were harvested by centrifugation at 3000 g for 10 min at 4°C and the pellet resuspended in 0.5 x the original culture volume of sterile cold $CaCl_2$ (100 mM). The cells were incubated on ice for 20 min before recentrifugation and resuspension in 0.1 x the original culture volume of 100 mM $CaCl_2$ containing 20% glycerol. The competent cells were aliquoted into sterile eppendorf tubes and stored at -80°C.

2.20.2. Standard transformation.

To the DNA solution on ice was added 100 µl TMC (10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$) and 200 µl competent cells. Incubation on ice was continued for 30 min before heat shocking the cells by incubation at 42°C for 2 min. 1 ml of LB was added to each transformation and non-selective expression of the transformed DNA was achieved in incubation at 37°C for 1 h before harvesting the cells and plating on selective media.

2.20.3. Low temperature transformation.

The procedure was identical to the standard transformation except that heat shock was at 30°C for 5 min before non-selective expression at 30°C.

2.21. Isolation of chromosomal DNA from *S. putrefaciens.*

The procedure used was a modification of that described in Maniatis *et al.* (1989). To minimise shearing of the chromosome during this procedure all pipetting was done using a wide bore pipette and mixing was by gentle inversion. *S. putrefaciens* was incubated aerobically overnight to stationary phase in 100 ml Wood and Baird broth. Cells were harvested by centrifugation at 4°C for 15 min at 10 000g then washed in 50 mM Tris-HCl (pH 8.0); 100 mM NaCl; 1 mM $MgCl_2$. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0); 5 mM EDTA; (10 ml) before addition of lysozyme (10 mg ml^{-1}) in 250 mM Tris-HCl pH 8.0 (0.5 ml) and incubated at 30°C for 30 min prior to addition of proteinase K (2 mg ml^{-1}) in STEP solution (0.5% SDS; 50 mM Tris-HCl pH7.5; 400 mM EDTA) (0.5 ml). Incubation was continued at 50°C for a further 30 min before removal of cell protein by phenol extraction which was repeated until a clear aqueous phase was obtained (centrifugation at 15°C for 15 min at 1000g). Nucleic acid was precipitated from the aqueous phase on addition of 3 M sodium acetate pH 5.0 (0.1 vol.) followed by ethanol (2 vol.). The precipitate of DNA and RNA was transferred with a sterile pasteur pipette into 5 ml 50 mM Tris-HCl (pH 7.5); 1 mM EDTA; containing 200 μg ml^{-1} DNase-free RNase then dissolved by gently rocking overnight at 4°C. RNase was removed by extraction with an equal volume of chloroform. Following centrifugation (15 min at 1000 g) DNA was precipitated from the aqueous phase on addition of 3 M sodium acetate (0.1 vol.) and ethanol (2 vol.). The precipitate was spooled out as before into 50 mM Tris-HCl (pH 7.5); 1 mM EDTA (2 ml) and dissolved at 4°C.

2.21.1. Estimation of DNA concentration and purity.

DNA concentration was estimated on the assumption that an A_{260} of 1.0 approximates to a DNA concentration of 50 $\mu g\ ml^{-1}$.

DNA purity was estimated by calculation of the A_{260}:A_{280} ratio. A value obtained of greater than 1.80 indicated DNA purity.

2.22. Generation of suitable fragments for cloning.

2.22.1. Pilot partial digest.

The pilot partial digest was used to determine the optimum digest time which yielded the maximum DNA fragments of the desired size.

The reaction mix containing chromosomal DNA (6 μg), bovine serum albumin (200 $\mu g\ ml^{-1}$) and appropriate enzyme buffer was preincubated to the required digestion temperature prior to addition of enzyme (0.5 units per μg DNA) to start the reaction. At measured time intervals after starting the reaction, a volume corresponding to 1 μg of DNA was removed from the reaction mix to eppendorf tubes on ice containing EDTA (final concentration of 20 mM). Samples were electrophoresed on agarose (0.5%) (section 2.17.1) to determine the optimum digest time.

2.22.2. Large scale partial digest.

This was essentially that of the pilot reaction scaled up for an increased DNA concentration (2 mg). The reaction was stopped by extraction with an equal volume of Tris buffered phenol followed by extraction with an equal volume of chloroform (both centrifugations at 15°C for 15 min at 1000 g). DNA was precipitated on addition of precooled ethanol (-20°C ; 2 vol.) and 3 M sodium acetate pH 5.0 (0.1 vol) followed by incubation at -20°C for 60 min, and

collected by centrifugation at 0°C for 10 min at 9000 g. The DNA pellet was washed in 70% chilled ethanol, vacuum dried then resuspended in TE.

2.22.3. Size fractionation of partial digest.

Continuous gradients of 10%-40% (w/v) sucrose (in 1 M NaCl; 20 mM Tris-HCl pH 8.0; 5 mM EDTA) were made in 15 ml polyallomer centrifuge tubes. Partially digested DNA (200 μg) (section 2.20.1.) was layered carefully on top of the gradient. The DNA was separated by centrifugation in a swing out rotor at 15°C for 24 h at 80 000 g and spun down with the brake off. The bottom of the tubes were pierced with a 19 gauge needle and aliquots (0.5 ml) collected in sterile eppendorf tubes. Efficient separation of the DNA was checked by electrophoresis of an aliquot of each alternate sample (20 μl) in agarose (0.5%). The λ-*Hind*III digested standard was diluted 1:1 with 40% sucrose before loading to compensate for the high sucrose content of the samples. Samples containing DNA of the desired size were pooled and sucrose removed by the addition of sterile distilled water (2 vol.) and precooled ethanol (0.1 the new vol.). DNA was precipitated at -20°C for 2 h, pelleted by centrifugation at 4°C for 30 min at 10 000 g, washed in ethanol (70%), vacuum dried, then resuspended in TE to a final concentration of approximately 100 μg ml^{-1}.

2.23. Construction and screening of a *S. putrefaciens* chromosomal gene library in pUN121.

2.23.1. Library construction.

Plasmid pUN121 was digested to completion with *Bcl*1 and ligated with 4-10 kb *Sau*3A chromosomal fragments from *S. putrefaciens* in the insert:vector ratio of 2:1 (section 2.16.). Aliquots of the ligation mix were transformed into *E. coli*

MM294. Transformant colonies containing inserts were selected on plates containing tetracycline and ampicillin (section 2.18.2.). Rapid plasmid preparations (section 2.15.1.) of 10 independant recombinants from each ligation mix were used to assess insert size. The library was pooled and used as a source of recombinant plasmid DNA (section 2.15.2.) which was then transformed into JRG780 (section 2.18.2.).

2.23.2. Complementation of the JRG780 *trpA* mutation.

JRG780 containing the pUN121 *S. putrefaciens* library was plated on to glycerol minimal medium lacking tryptophan at a density of $2x10^3$ colonies per plate. Putative positive colonies were selected by incubation at 28°C for 48-72 h. Plasmid DNA was isolated from possible complementing colonies, retransformed into a clean host background and reselected as before.

2.23.3. Complementation of the JRG780 *frdA* mutation.

JRG780 containing the pUN121 library was plated on to glyerol fumarate minimal medium plates as above. Putative clones were selected by incubation in an anaerobic jar at 28°C for 48-72 h prior to retransformation and reselection.

Putative positive clones were further analysed as detailed below. A single colony was grown to mid-exponential phase (A_{600} of 0.4) in 60 ml LB containing tetracycline. A sample (1 ml) was removed for electrophoresis and Western blotting (section 2.12). The remainder was added to fresh broth (200 ml) containing 0.4 % glucose (final concentration) and 0.04 M fumarate pH 7, in a 250 ml capacity flask and incubated statically at 28°C for 16 h. Cultures were harvested and washed twice in buffer (10 mM Tris-HCl pH 8.0) before resuspension to 0.25 g ml^{-1} (wet weight) in buffer and broken by sonication (2 x

1 min with a break of 1 min). The lysate was assayed for fumarate reductase activity (section 2.13.1). A sample was retained for electrophoresis (section 2.10) and Western blotting (section 2.12).

2.24. Construction and screening of a *S. putrefaciens* chromosomal gene library in the pEX vectors.

2.24.1. Library construction.

Plasmid DNA was digested to completion with *Bam*H1, phosphatased, and ligated to 0.5-4 kb chromosomal fragments from *S. putrefaciens* (section 2.16.). The vector : insert ratio was optimised to yield a recombinant frequency of 80%-90%. The ligation mix was transformed into competent *E.coli* MM294 containing plasmid pCI857 (Figure 2.1.) and transformants selected at 30°C on plates containing ampicillin and kanamycin. The recombinant frequency of each ligation was assessed by selecting 10 independent transformants from which plasmid DNA was isolated, digested with *Pst*I and sized by comparison with a non recombinant control. The library initially contained 20 000 independent recombinants which were pooled and stored at -80°C.

2.24.2. Primary screen.

The library was plated on L-agar containing ampicillin and kanamycin at a density of $2x10^3$ colonies per plate and incubated at 30°C for 20 h. Colonies were replica plated on to dry nylon filters (Amersham Hybond N) and identification marks pricked through the filter on to the agar surface. Filters were placed with the colony side up on to 2 layers of Whatman 3MM filter paper presoaked in Luria broth containing ampicillin and kanamycin, and incubated at 42°C for 2 h. Filters were removed, colony side uppermost, to

petri dishes containing 2 layers of Whatman 3MM filter paper soaked in 5% SDS. Plates were stacked in a 95°C oven (with a heavy weight on top to prevent warping) and incubated for 25 min. The SDS was removed from the filters by electroblotting in transfer buffer (50 V for 1 h) with the colonies orientated towards the negative electrode. The filters were washed individually for 5 min in TBS followed by 10 min in TBS containing DNase (10 mg ml^{-1}). Excess moisture was removed from the filters by blotting on Whatman 3MM filter paper before blocking overnight in 20% milk powder made in TBS.

Filters were developed individually in petri dishes as for Western blots. Colonies developing more strongly than the background were isolated then subjected to a further primary screen as before.

2.24.3. Secondary screen of putative positive clones.

The secondary screen was a more detailed analysis of putative positive recombinants to determine the size of hybrid protein and whether it would react with flavocytochrome specific antibody by Western blotting. Single colonies were incubated aerobically in Luria broth containing ampicillin and kanamycin (20 ml) at 30°C to reach an A_{600} of 0.4. A sample was removed (10 ml) of which 5 ml was used as a source of plasmid DNA (section 2.15.1.) and 5 ml concentrated to 200 μl in sample buffer (section 2.12.), and stored at -20°C. The remaining 10 ml was incubated in a shaking water bath at 42°C for 90 min then treated as the uninduced culture. Equal loadings of each sample (uninduced and induced) were electrophoresed (section 2.10.) prior to staining (section 2.11.) and Western Blotting (section 2.12.).

CHAPTER 3

PROTEIN PURIFICATION

AND

FLAVOCYTOCHROME C ENZYME ACTIVITY

3.1. Purification of flavocytochrome c.

Periplasm was isolated from *S. putrefaciens* cultured microaerophilically in the presence of fumarate to induce synthesis of cytochromes involved in electron transfer during oxygen limitation. Flavocytochrome c was previously shown to be induced to the greatest degree with fumarate as terminal electron acceptor (Morris, 1987) and was subsequently purified from the periplasm according to the following protocol.

The first stage of purification involved chromatography on DEAE Sepharose as this chromatographic medium effectively separated periplasmic proteins, in particular the periplasmic cytochromes (Morris, 1987). The profile obtained following elution (Figure 3.1.) showed at least three major peaks of cytochrome with various smaller peaks of protein. To isolate the peak corresponding to flavocytochrome c, fractions contributing to the three cytochrome peaks were pooled separately and the proteins in the samples resolved by SDS-PAGE. Subsequent haem and silver staining of the gels showed flavocytochrome c to be located in the first major cytochrome peak (Plates 3.1. and 3.2.), confirming earlier elution profiles of periplasm from DEAE Sepharose (Morris, 1987) from which flavocytochrome c was the first major cytochrome eluted at an NaCl molarity of 0.16 M. Purification was continued by sequential application of the flavocytochrome c-containing fraction to hydroxyapatite, phenyl sepharose and gel filtration matrices. A single peak of cytochrome and major peak of protein was observed on development of each column (Figures 3.2.-3.4.). Purity of the cytochrome peak was monitored at each stage of the purification by SDS-PAGE followed by silver staining (Plate 3.1.). The cytochrome contributing to this peak was confirmed as flavocytochrome c by SDS-PAGE followed by haem staining (Plate 3.2.) and was the only cytochrome remaining in the sample following chromatography on

Figure 3.1. Elution profile of periplasm from fumarate grown *S. putrefaciens* on DEAE Sepharose.

Periplasm was obtained from *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) and bound to DEAE Sepharose equilibrated with 10mM Tris-HCl pH 8.4 (T-buffer). The column was washed with approximately one column volume of this buffer to remove unbound material, then developed by application of an increasing linear gradient of 0-0.5 M NaCl in T-buffer. Loading of material, washing, and elution of the sample were carried out at a constant flow rate of 8 ml h^{-1} with fractions of 4 ml collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

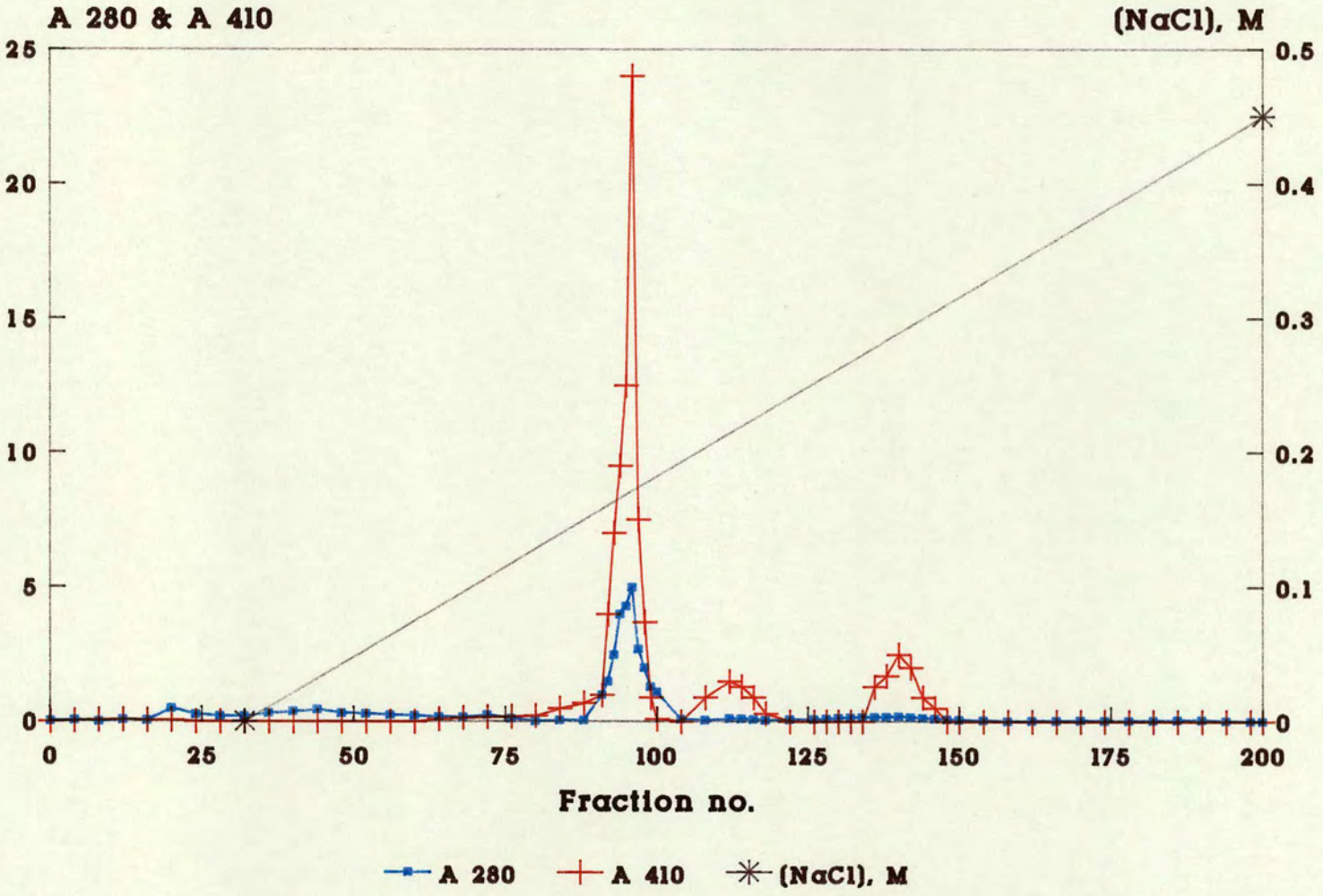

A 280 & A 410
(NaCl), M
25
20
15
10
5
0
0.5
0.4
0.3
0.2
0.1
0
0
25
50
75
100
125
150
175
200
Fraction no.
A 280
A 410
(NaCl), M

Figure 3.1.

Plates 3.1. and 3.2. Silver and haem stain of flavocytochrome c purification : method I.

Periplasm was obtained from *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) and flavocytochrome c purified by the sequential application of the periplasm to DEAE Sepharose, hydroxyapatite, phenyl sepharose and Sephacryl S-300. The purity of the flavocytochrome-containing fraction following each stage was assessed by SDS-PAGE (7.5-15% resolving gel). Approximately 10 μg of each sample was electrophoresed prior to silver staining (Plate 3.1.), and 30 μl of a sample corresponding to an A_{410} of 0.25 for haem staining (Plate 3.2.).

Lane	Plate 3.1.	Lane	Plate 3.2.
1	Protein standards	1	Sephacryl S-300 fraction
2	Fumarate periplasm	2	phenyl sepharose fraction
3	DEAE Sepharose fraction	3	hydroxyapatite fraction
4	hydroxyapatite fraction	4	DEAE Sepharose fraction
5	phenyl sepharose fraction	5	Fumarate periplasm
6	Sephacryl S-300 fraction	6	Horse heart cytochrome-c

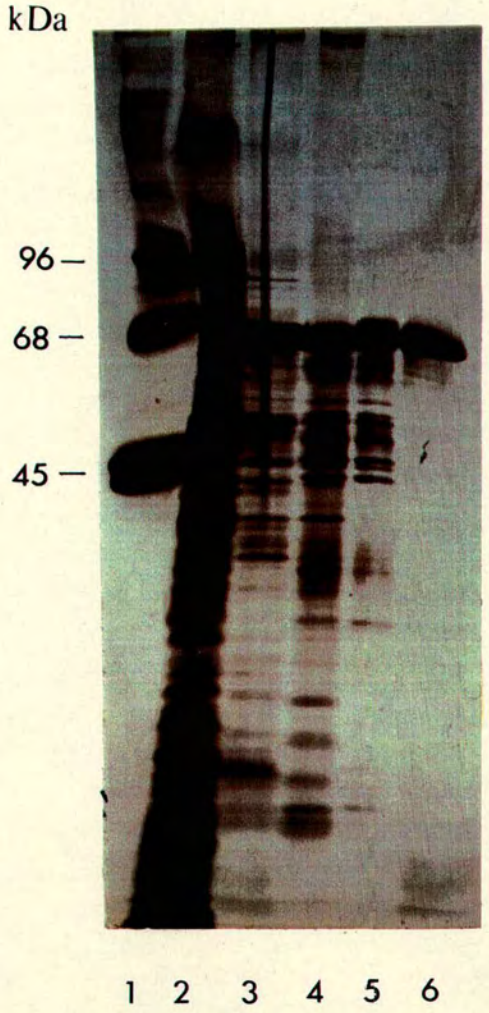


Plate 3.1.

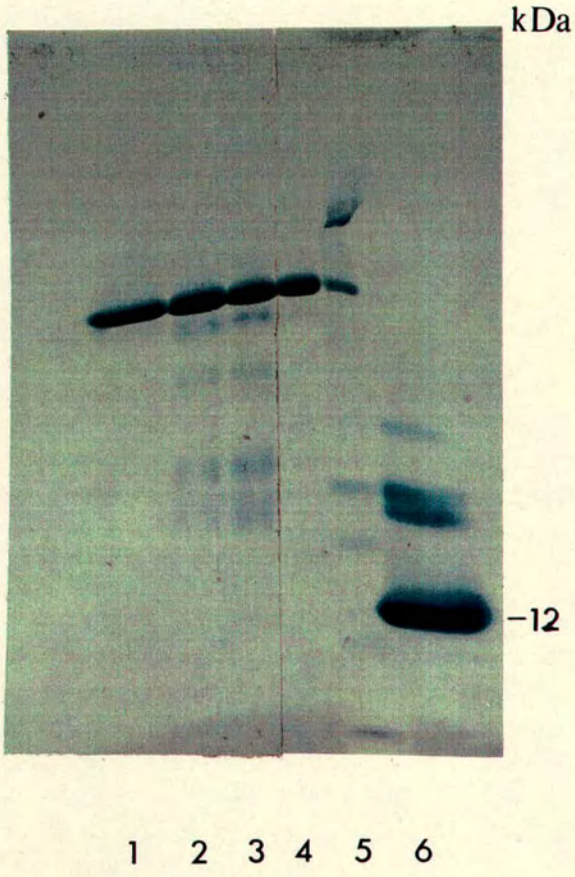


Plate 3.2.

Figure 3.2. Elution profile of DEAE Sepharose flavocytochrome c fraction on hydroxyapatite.

The flavocytochrome c-containing fraction collected following chromatography on DEAE Sepharose was bound to hydroxyapatite equilibrated in T-buffer. The column was washed with T-buffer (one column volume) then developed by application of an increasing linear gradient of 0-0.5 M K_2HPO_4 in T-buffer. Loading of material, washing, and elution of the sample were carried out at a constant flow rate of 8 ml h^{-1} with fractions of 4 ml collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

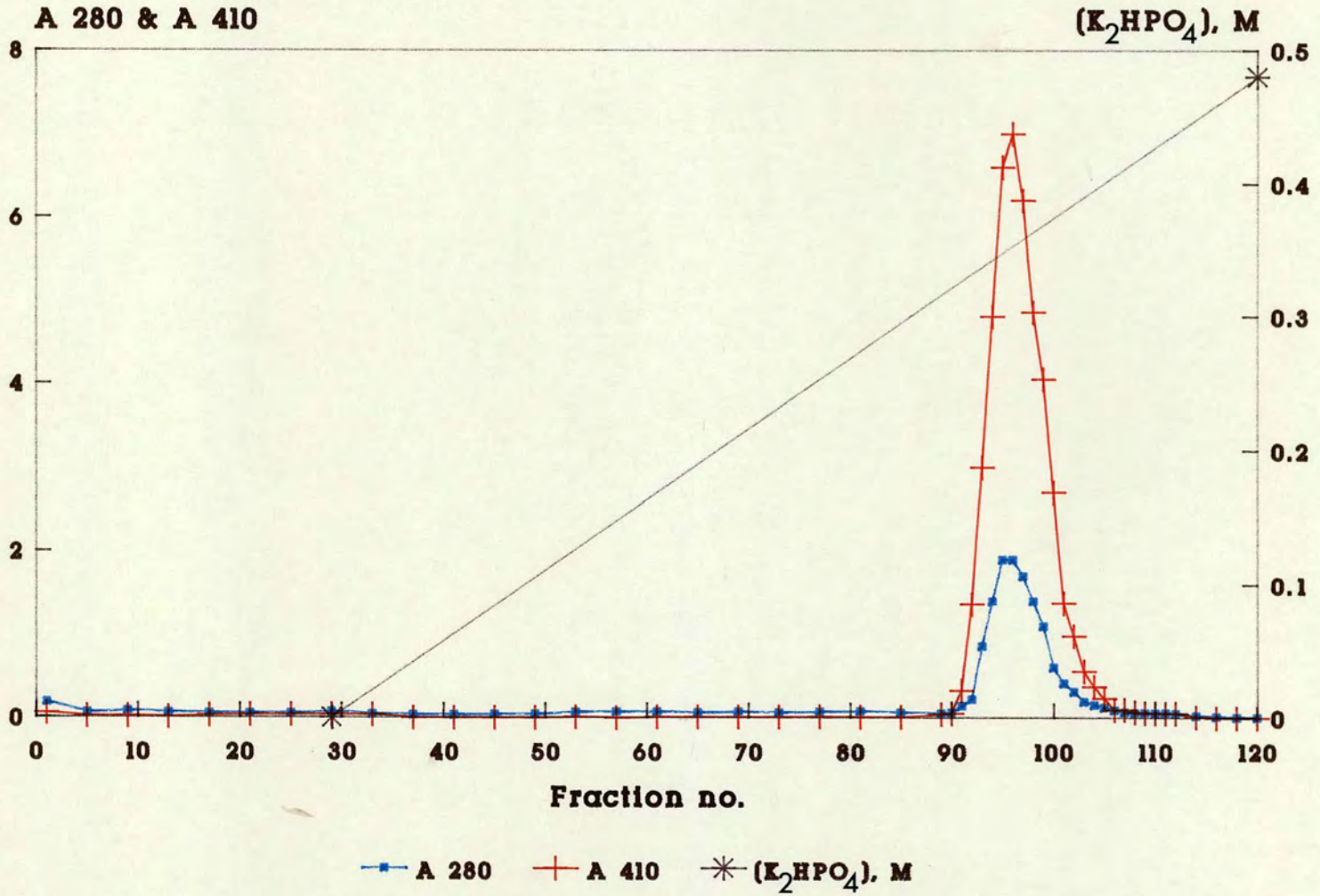

A 280 & A 410
(K2HPO4), M
8
6
4
2
0
0.5
0.4
0.3
0.2
0.1
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Fraction no.
A 280
A 410
(K2HPO4), M

Figure 3.2.

Figure 3.3. Elution profile of hydroxyapatite flavocytochrome c fraction on phenyl sepharose.

The flavocytochrome c-containing fraction collected following chromatography on hydroxyapatite was bound to phenyl sepharose equilibrated in T-buffer containing 2 M NaCl. The column was washed with T-buffer (plus 5 mM EDTA) containing 2 M NaCl (one column volume) then developed by application of a decreasing linear gradient of 2 M-0 M NaCl in T-buffer containing EDTA (5 mM). Loading of material, washing, and elution of the sample were carried out at a constant flow rate of 12 ml h^{-1} with 4 ml fractions collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

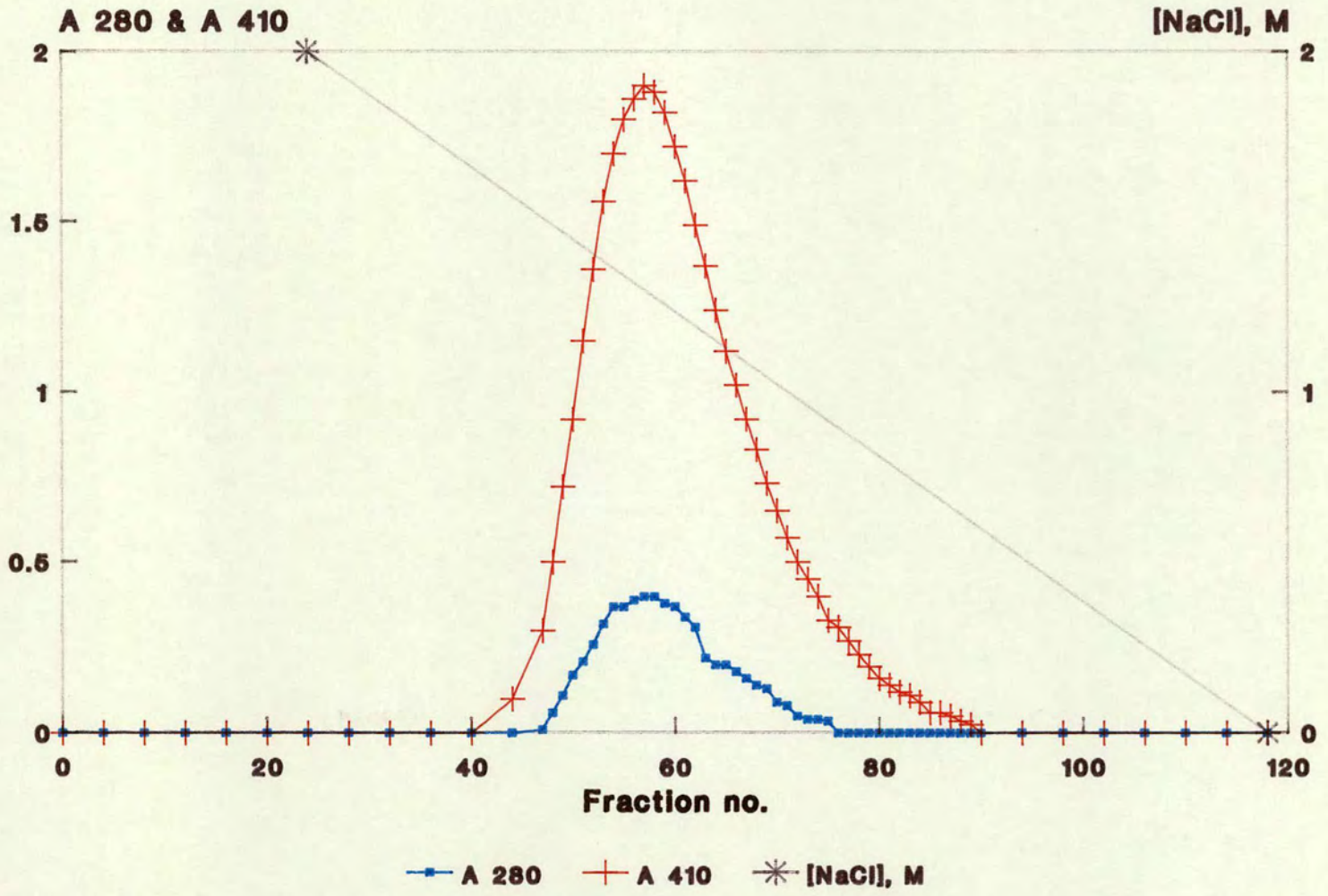

A 280 & A 410
[NaCl], M
2
1.5
1
0.5
0
2
1
0
0
20
40
60
80
100
120
Fraction no.
A 280
A 410
[NaCl], M

Figure 3.3.

Figure 3.4. Elution profile of phenyl sepharose flavocytochrome c fraction on Sephacryl S-300.

The flavocytochrome c-containing fraction collected following chromatography on phenyl sepharose was concentrated to a volume of 4 ml by dialysis against solid PEG 6000 at $4^{o}C$ then applied to Sephacryl S-300 superfine equilibrated in T-buffer (plus 5mM EDTA) containing 0.2 M NaCl. Development was achieved by passing equilibration buffer through the column at a constant flow rate of 12 ml h^{-1} with 4 ml fractions collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

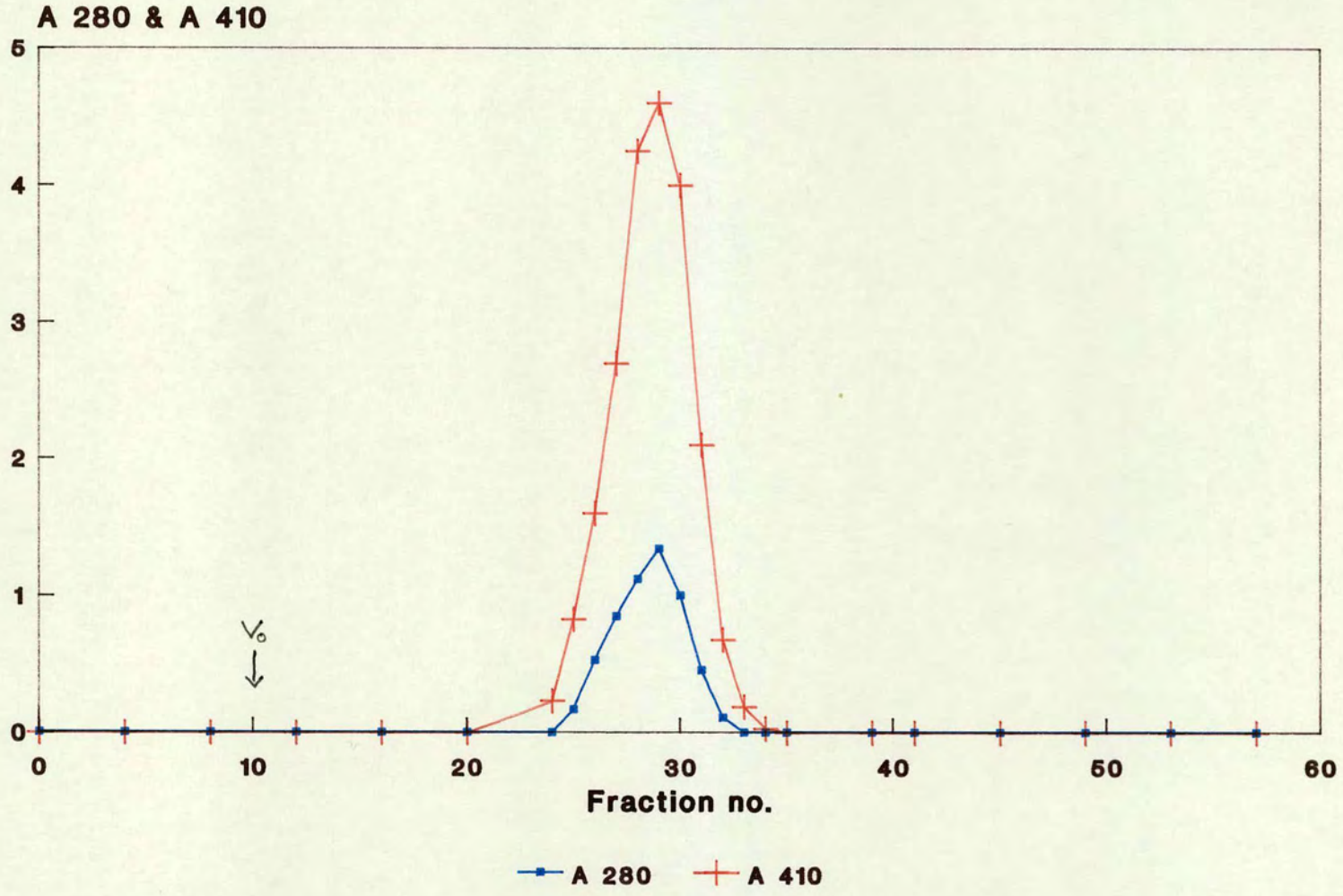


Figure 3.4.

DEAE Sepharose. The other proteins co-eluting with flavocytochrome c were removed during this purification procedure. Following gel filtration the protein was judged to be pure to homogeneity as determined by both silver and haem stains (Plates 3.3. and 3.4.). Due to the inability of the silver stain to detect all protein types (Biorad bulletin 1089), a sample of the purified flavocytochrome was stained with Kenacid Blue. No protein other than flavocytochrome c was detected by this stain (results not presented).

One problem encountered during the latter stages of purification, following chromatography on hydroxyapatite, was slight degradation of flavocytochrome c (Lane 3, Plate 3.2.). Further proteolysis seemed to be prevented by the presence of EDTA in the T-buffer at all remaining stages of purification, and the proteolytic fragments were removed by chromatography on Sephacryl S-300. Throughout the purification, protein concentrations were measured by the Folin Ciocalteau assay (section 2.8.) following each purification stage and the A_{410}:A_{280} ratio was used as a crude estimate of purity. The overall scheme of purification is presented in Table 3.1. and discussed fully in section 3.3.

3.2. Development of a new flavocytochrome c purification method

It was decided to develop a new procedure for purification of the flavocytochrome as the existing procedure, although effective in yielding a substantial quantity of pure protein, was relatively lengthy involving chromatography of the flavocytochrome-containing material through four different column matrices, and also gave slight protein degradation. A purification method was sought therefore which 1) reduced processing time of the flavocytochrome fraction and 2) minimised protein degradation.

Plates 3.3. and 3.4. Silver and haem stain of purified flavocytochrome c.

Purified flavocytochrome c was electrophoresed on SDS-PAGE (7.5-15% resolving gel). Approximately 10 μg of protein was loaded for silver staining (Plate 3.3.) and a cytochrome concentration corresponding to an A_{410} of 0.25 in 30 μl for haem staining (Plate 3.4.).

Lane	Plate 3.3.	Lane	Plate 3.4.
1	Protein standards	1	Horse heart cytochrome-c
2	Fumarate periplasm	2	Fumarate periplasm
3	Purified flavocytochrome c	3	Purified flavocytochrome c

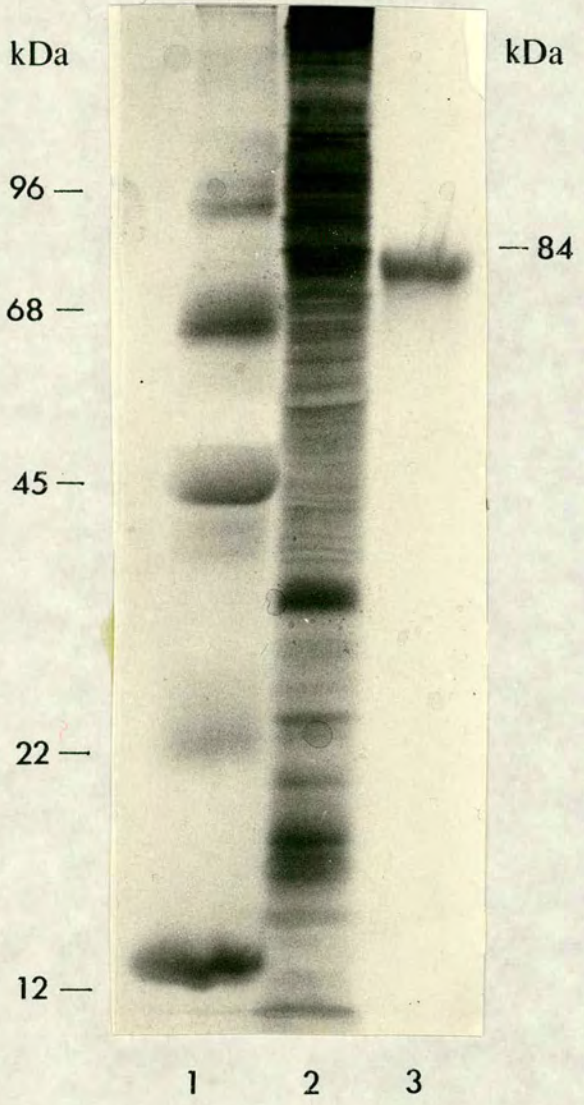


Plate 3.3.

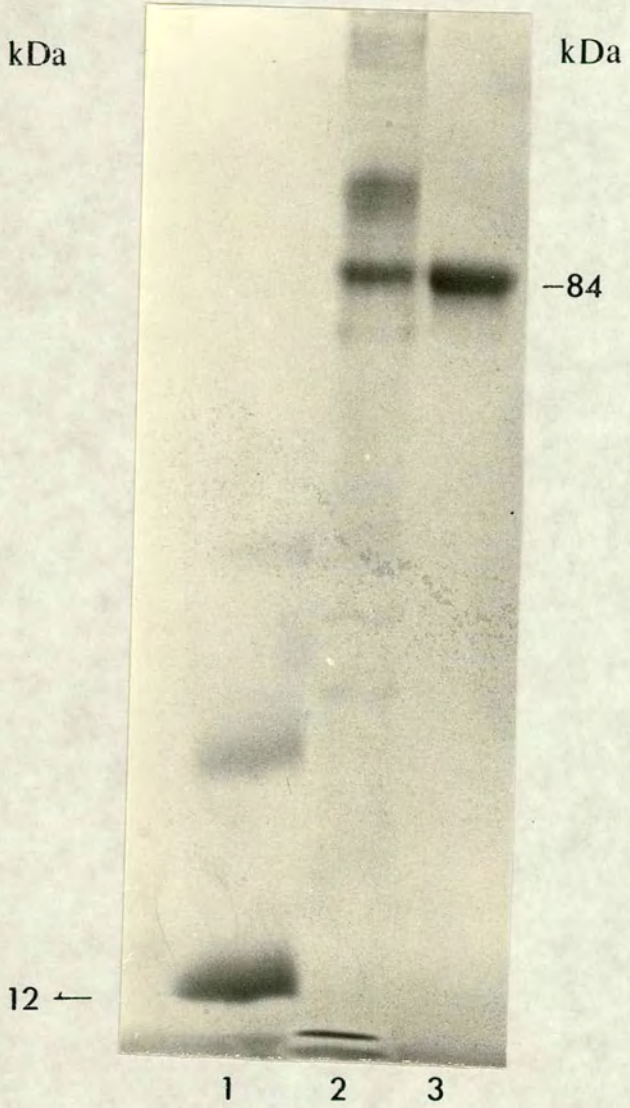


Plate 3.4.

Table 3.1. Purification of flavocytochrome c : method I.

(cytochrome determination)

Flavocytochrome c was purified from periplasm prepared from *S. putrefaciens* grown microaerobically in the presence of fumarate according to the procedure in Table 3.1. The protein concentration following each purification stage was determined by the Folin-Ciocalteau protein assay (section 2.8.), and haem content was estimated from the absorbance at 410 nm assuming an extinction coefficient of 730 mM^{-1} cm^{-1} for flavocytochrome haem (Morris, 1987).

Purification of flavocytochrome c : method I.

(cytochrome determination)

Source	A_{410U} (% Yield)	Total Protein (mg) (% Yield)	nmol haem (% Yield)	nmol haem/mg protein	Purification (fold)
Periplasm	832.5 (100)	99.9 (100)	1140 (100)	11.4	1
Ion Exchange (DEAE Sepharose)	320 (38)	33.94 (34)	601 (53)	17.7	1.55
Hydroxyapatite	134.4 (16.1)	9.6 (9.7)	197 (17)	20.5	1.8
Phenyl Sepharose	99.3 (11.9)	3.9 (3.89)	136 (11.9)	34.8	3.05
Gel Filtration (Sephacryl S-300)	62.4 (7.5)	4 (3.9)	85.5 (7.5)	21.4	1.87

3.2.1. Precipitation of periplasm by ammonium sulphate.

The possibility of using selective ammonium sulphate fractionation as the first purification step following extraction of periplasm was investigated. Periplasm was obtained from microaerobically grown *S. putrefaciens* with fumarate as terminal electron acceptor, and fractionated by the sequential addition of solid ammonium sulphate. Fractions of 0-30%, 30-40%, 40-50%, 50-60%, 60-80% and 80-100% saturation were collected. On the basis of earlier work, EDTA was added to all buffers to minimise the occurrence of any proteolysis.

The total protein content of the supernatant and pellet obtained from each fraction was measured and the data are presented in Figure 3.5. The chart shows that the majority of protein (60%) was precipitated in the 60-80% fraction, with approximately 30% of the periplasmic protein precipitated in fractions from 0-60% and residual protein (about 10%) precipitated at 80-100%. Very little protein remained unprecipitated.

The location of the flavocytochrome in the ammonium sulphate fractions was confirmed by SDS-PAGE of the precipitates and supernatants from each fraction followed by silver and haem staining (Plates 3.5.-3.8.). Haem staining showed the flavocytochrome to be precipitated mostly at 60-80% and 80-100% saturation (Plate 3.6.), although some minor precipitation did occur at lower concentrations. All other periplasmic cytochromes were precipitated at fractions from 0-60% with the exception of a cytochrome migrating at the gel dyefront which co-precipitated with flavocytochrome c at 60-80% saturation. Silver staining showed a variety of proteins co-precipitating with the flavocytochrome in the 60-80% and 80-100% fractions although flavocytochrome c was the major protein present.

Figure 3.5. Precipitation of periplasmic protein from fumarate-grown *S. putrefaciens* by ammonium sulphate.

Periplasm obtained from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) was sequentially fractionated by the addition of solid ammonium sulphate (section 2.7). The distribution of periplasmic protein following fractionation was determined by measuring the protein content (by the Folin-Ciocalteau protein assay) of supernatant and precipitate fractions.

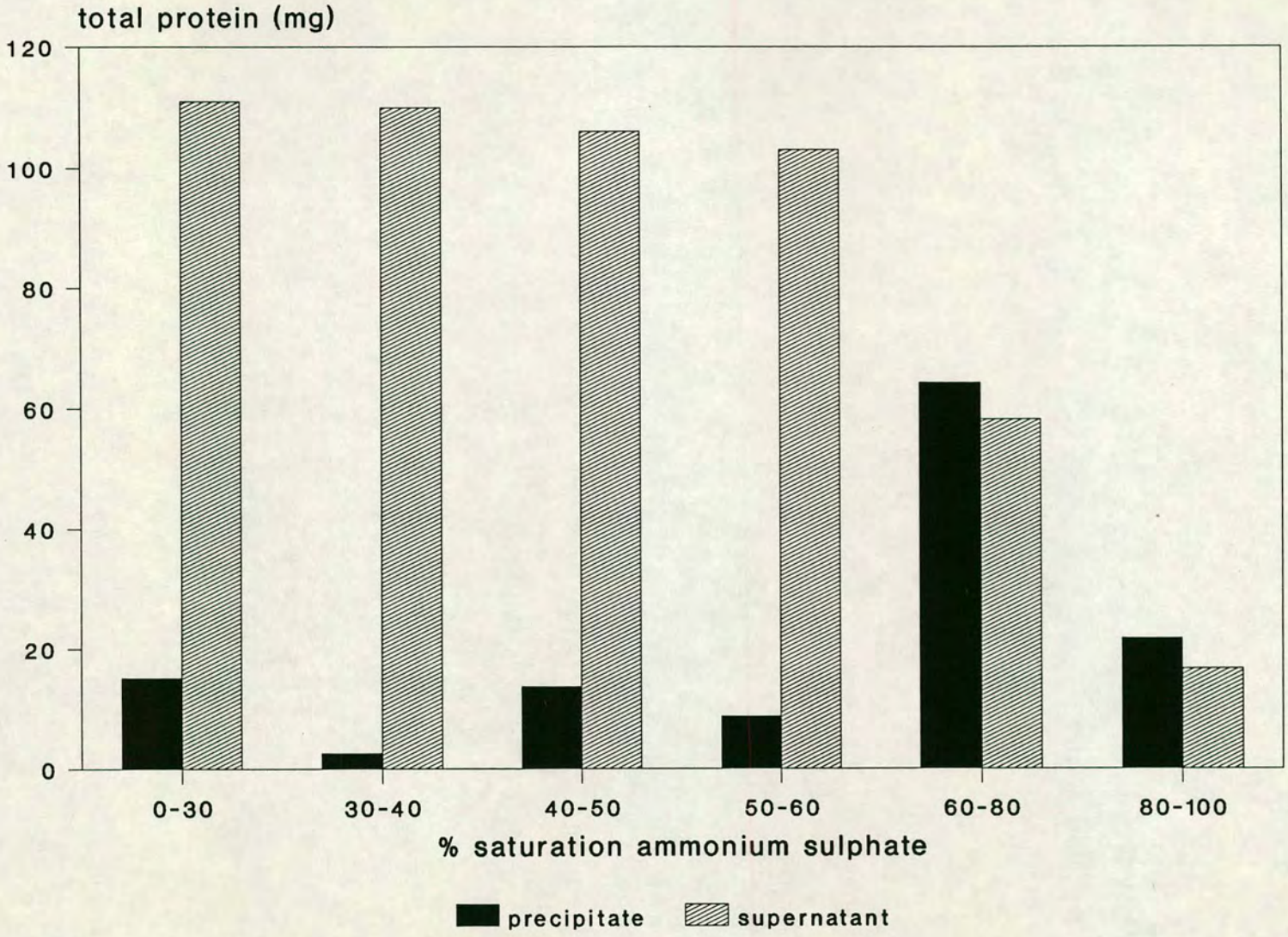

total protein (mg)
120
100
80
60
40
20
0
0-30
30-40
40-50
50-60
60-80
80-100
% saturation ammonium sulphate
precipitate
supernatant

Figure 3.5.

Plates 3.5. and 3.6. Silver and haem stain of precipitate fractions following ammonium sulphate precipitation of periplasm.
Periplasm obtained from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5.) was sequentially fractionated by the addition of solid ammonium sulphate (section 2.7.). Precipitated protein from each fraction was dialysed against 10 mM Tris-HCl pH 8.4, 5 mM EDTA to remove ammonium sulphate prior to electrophoresis on SDS-PAGE (7.5-15%) resolving gel. Approximately 10 μg of protein from each fraction was electrophoresed prior to silver staining (Plate 3.5.), and 30 μl of an A_{410} of 0.25 from each fraction electrophoresed before haem staining (Plate 3.6.).

Lane	Plate 3.5.	Lane	Plate 3.6.
1	Protein standards	1	Horse heart cytochrome-c
2	0-30%	2	0-30%
3	30-40%	3	30-40%
4	40-50%	4	40-50%
5	50-60%	5	50-60%
6	60-80%	6	60-80%
7	80-100%	7	80-100%
8	Fumarate periplasm	8	Fumarate periplasm
9	Purified flavocytochrome c	9	Purified flavocytochrome c

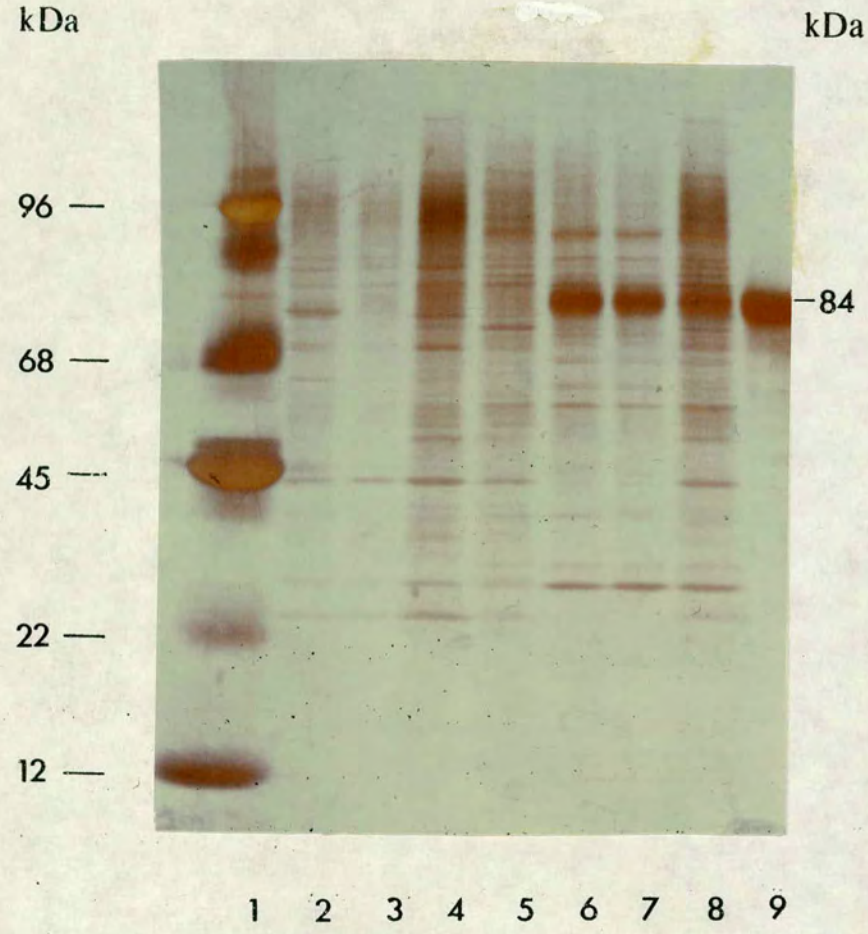


Plate 3.5.

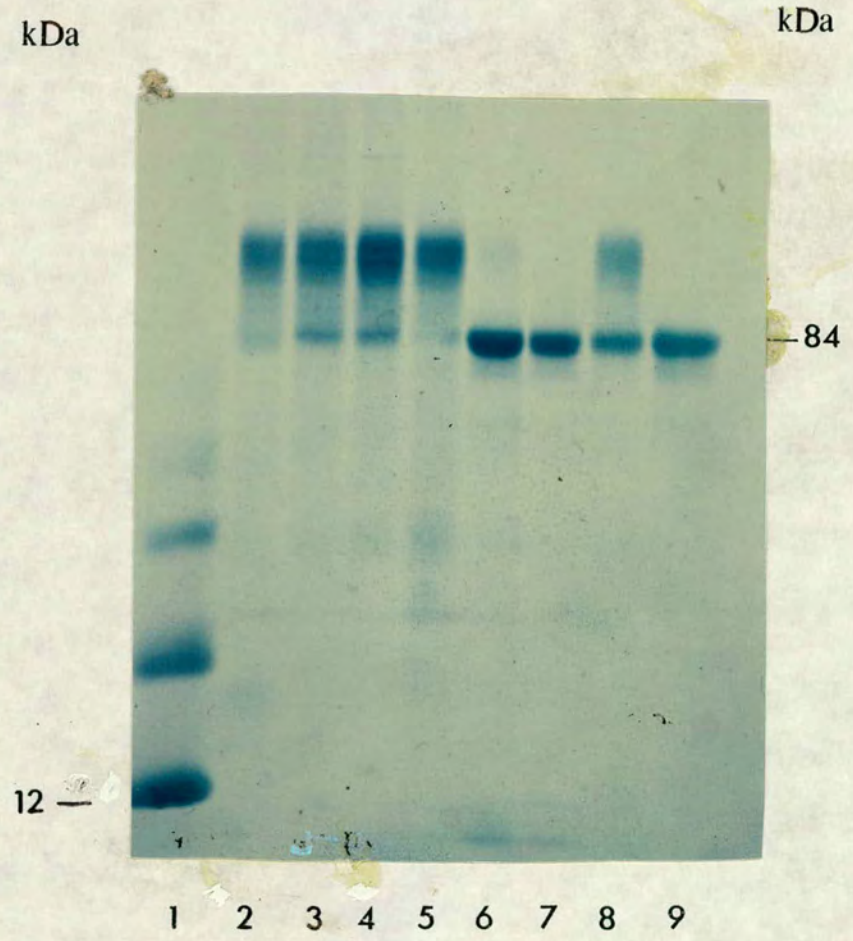


Plate 3.6.

Plates 3.7. and 3.8. Silver and haem stain of supernatant fractions following ammonium sulphate precipitation of periplasm.

Periplasm from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5.) was sequentially fractionated by the addition of solid ammonium sulphate (section 2.7.). The supernatant from each fraction was dialysed against 10 mM Tris-HCl pH 8.4, 5 mM EDTA to remove ammonium sulphate prior to electrophoresis on SDS-PAGE (7.5-15%) resolving gel. Approximately 10 μg of protein from each fraction was electrophoresed prior to silver staining (Plate 3.7.), and 30 μl of an A_{410} of 0.25 from each fraction electrophoresed before haem staining (Plate 3.8.).

Lane	Plate 3.7.	Lane	Plate 3.8.
1	Protein standards	**1**	Horse heart cytochrome-c
2	0-30%	**2**	0-30%
3	30-40%	**3**	30-40%
4	40-50%	**4**	40-50%
5	50-60%	**5**	50-60%
6	60-80%	**6**	60-80%
7	80-100%	**7**	80-100%
8	Fumarate periplasm	**8**	Fumarate periplasm
9	Purified flavocytochrome c	**9**	Purified flavocytochrome c

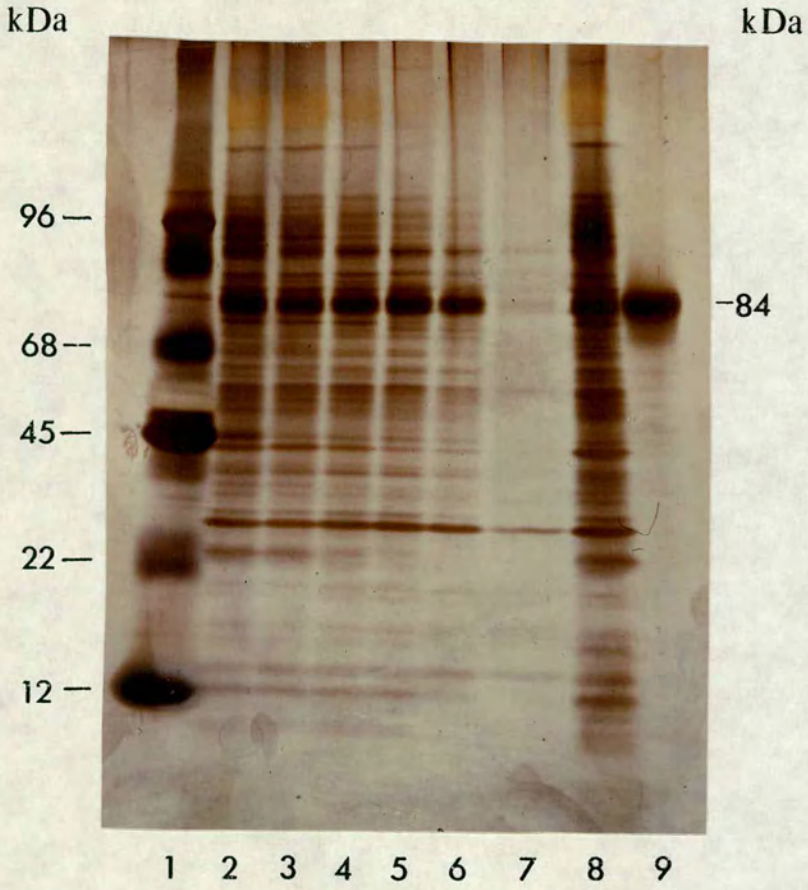


Plate 3.7.

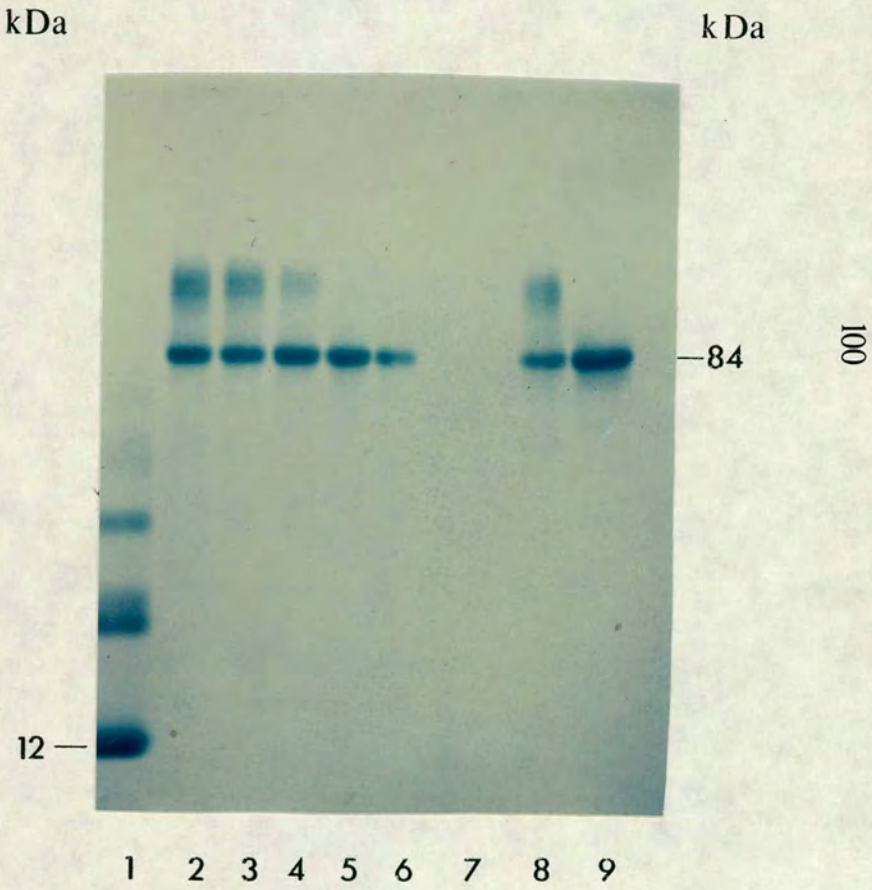


Plate 3.8.

Total and specific fumarate reductase activities were determined for each precipitate and supernatant fraction (Figures. 3.6. and 3.7.). Total activity determination showed that most enzyme activity (70%) was located in the 60-80% fraction with very little activity present in precipitates from 0-60% (10% in total). All remaining activity was precipitated in the 80-100% fraction and no detectable activity remained unprecipitated. The highest specific activity was detected in the 60-80% precipitate giving a value which was 5-fold higher than in the 0-60% precipitate and 2.5-fold higher than the 80-100% precipitate. This confirmed earlier observations that flavocytochrome c was precipitated at 60-80% saturation. The decrease in specific activity of supernatant fractions parallelled clearly the increase in specific activity of precipitate fractions. There is evidence that flavocytochrome c has the physiological ability to act as a fumarate reductase (investigated in more detail in section 3.5.), which is indicated in this initial purification stage, where the level of fumarate reductase activity correlates closely with the amount of flavocytochrome c present in the fraction.

In this purification, the 60%-80% and 80%-100% fractions were pooled to be used for subsequent stages.

3.2.2. Ion exchange chromatography of the pooled $(NH_4)_2SO_4$ precipitate.

The pooled 60-100% precipitate was applied to a DEAE Sepharose column as described in section 2.6.1., following resuspension in buffer (10 mM Tris-HCl pH 8.4, 5 mM EDTA) and dialysis against the same to remove ammonium sulphate. The elution profile (Figure 3.8.) showed two peaks of cytochrome : the major peak of cytochrome and protein eluted at approximately 0.16 M NaCl with the smaller peak of cytochrome being eluted towards the end of the run. Comparison of this profile with the ion exchange profile from the

Figure 3.6. Precipitation of periplasmic fumarate reductase activity from *S. putrefaciens* by ammonium sulphate.

Periplasm obtained from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) was sequentially fractionated by the addition of solid ammonium sulphate (section 2.7). The fumarate reductase activity of each precipitate and supernatant fraction was measured.

* Total activity = μ mol MV^+ oxidised min^{-1} x mg total protein in sample.

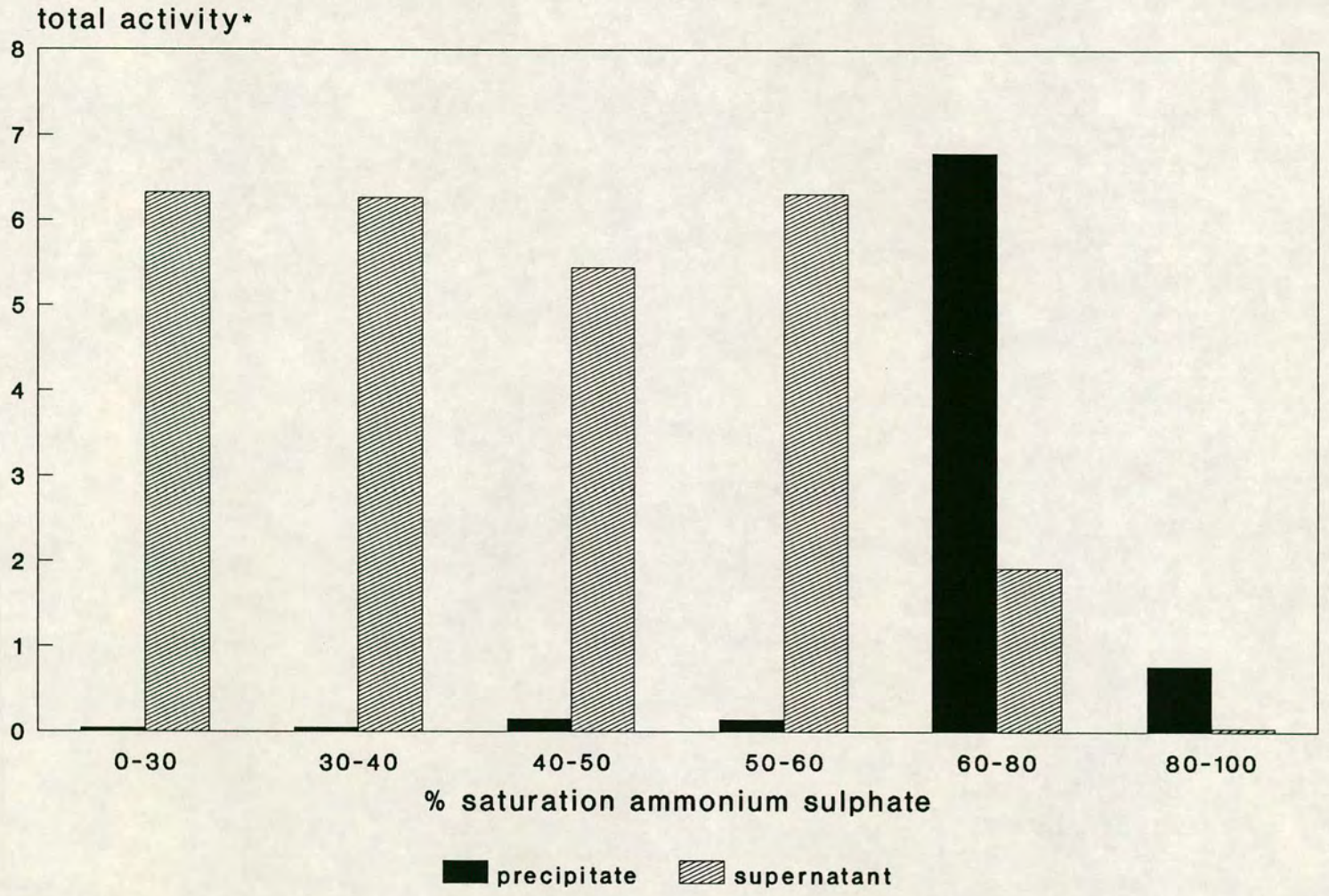

total activity*
8
7
6
5
4
3
2
1
0
0-30
30-40
40-50
50-60
60-80
80-100
% saturation ammonium sulphate
precipitate
supernatant

Figure 3.6.

Figure 3.7. Fumarate reductase specific activity following ammonium sulphate precipitation of periplasm from fumarate-grown *S. putrefaciens*.

Periplasm obtained from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) was sequentially fractionated by the addition of solid ammonium sulphate (section 2.7). The fumarate reductase specific activity was determined for each precipitate and supernatant fraction.

* Specific activity = μ mol MV^{++} oxidised min^{-1} mg $protein^{-1}$.

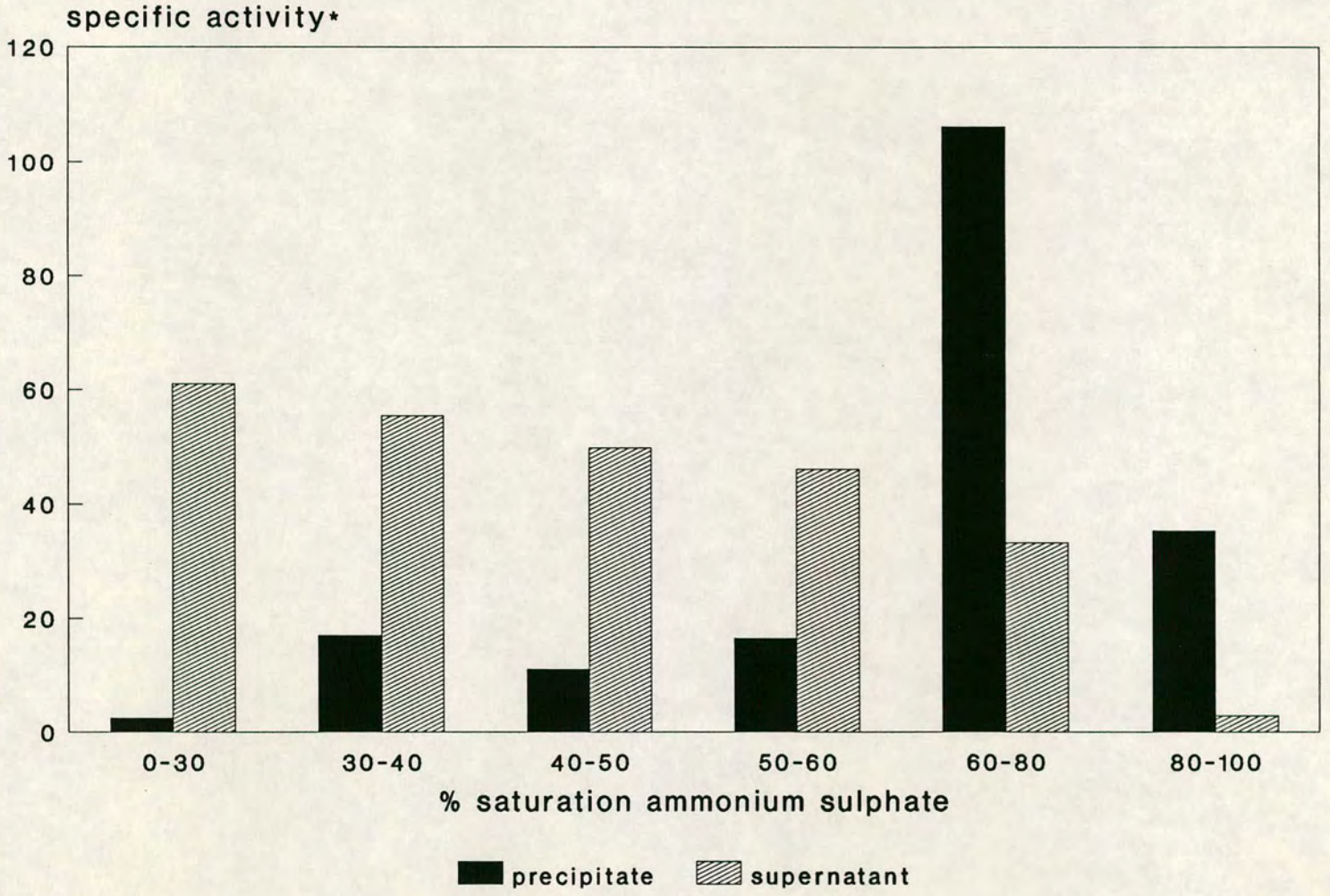

specific activity*
120
100
80
60
40
20
0
0-30
30-40
40-50
50-60
60-80
80-100
% saturation ammonium sulphate
precipitate
supernatant

Figure 3.7.

Figure 3.8. Elution profile of 60-100% ammonium sulphate precipitate on DEAE Sepharose.

The pooled, dialysed 60-100% ammonium sulphate precipitate fractionated from cells of *S. putrefaciens* grown microaerobically in the presence of fumarate (section 2.5) was bound to DEAE Sepharose equilibrated with 10mM Tris-HCl pH 8.4 (T-buffer) containing EDTA (5 mM). The column was washed with approximately one column volume of this buffer to remove unbound material, then developed by application of an increasing linear gradient of 0-0.5 M NaCl in T-buffer. Loading of material, washing, and elution of the sample were carried out at a constant flow rate of 8 ml h^{-1} with 4 ml fractions collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

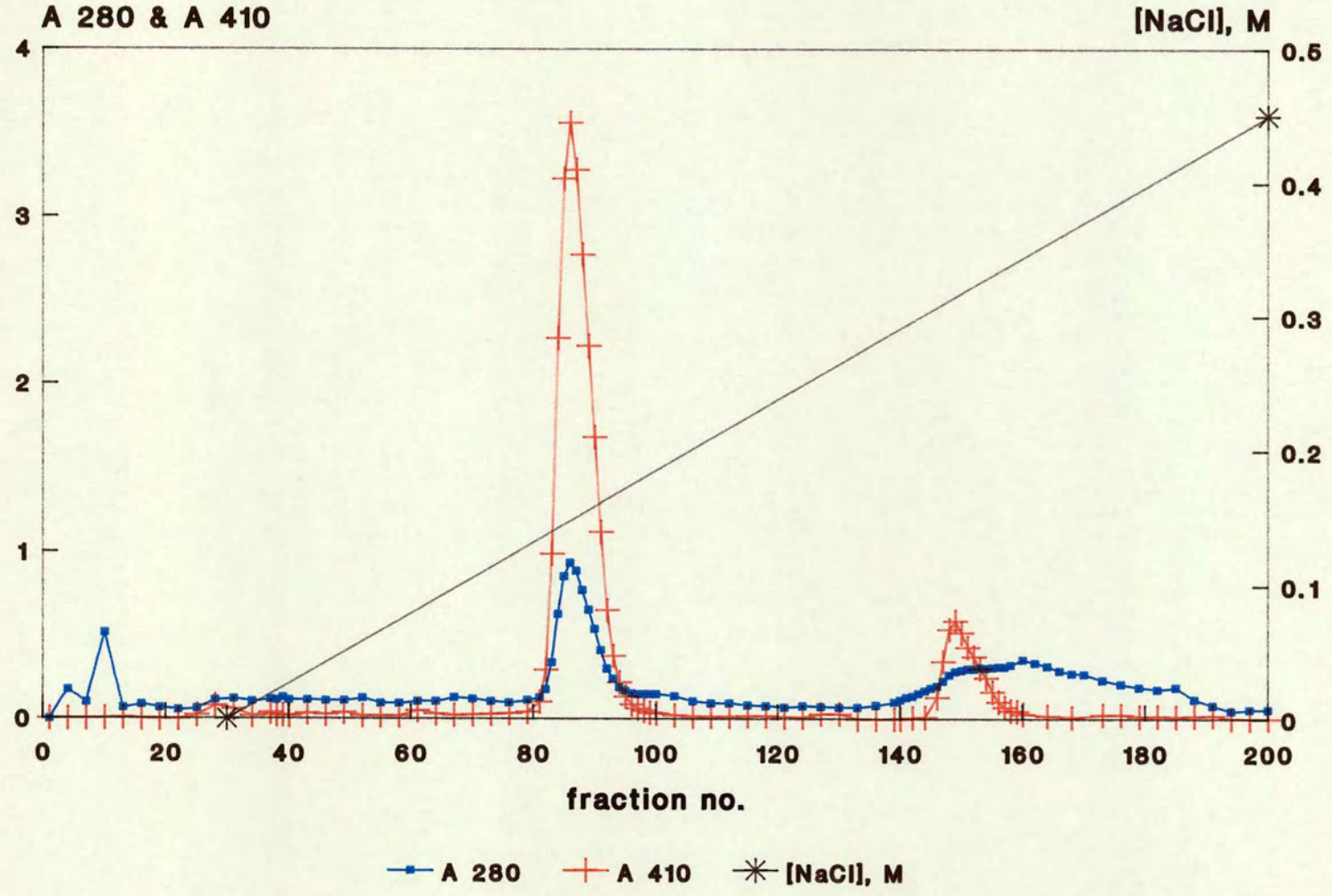

A 280 & A 410
[NaCl], M
4
3
2
1
0
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
200
fraction no.
A 280
A 410
[NaCl], M

Figure 3.8.

previous purification showed the removal of an additional cytochrome by ammonium sulphate precipitation. The proposed reasons for this are discussed later in section 3.3. Fractions contributing to the major peak of cytochrome were pooled. SDS-PAGE followed by silver staining showed flavocytochrome c to be the major protein in the sample although at least 6 additional minor proteins were detected. (Plate 3.9.). Haem staining (Plate 3.10.) showed a single cytochrome corresponding to flavocytochrome c.

3.2.3. Hydroxyapatite chromatography of DEAE Sepharose fraction.

The flavocytochrome-containing fraction from ion exchange chromatography was applied to hydroxyapatite. Following development of the column, the elution profile (Figure 3.9.) showed a single cytochrome and major protein peak eluting at approximately 0.34 M phosphate. A smaller protein peak was observed immediately on application of the phosphate gradient to the column and a low level of background protein was present throughout elution. Fractions containing the cytochrome peak were pooled and electrophoresed. Following this, silver and haem stains showed a single protein and haem-staining band corresponding to flavocytochrome c (Plates 3.9. and 3.10.). Although 2 bands corresponding to flavocytochrome c were observed in lanes 2, 3 and 4 of Plate 3.10. these were thought to be due to slight degradation of these samples on storage at 4^{o}C, as purified flavocytochrome c (Lane 5, Plate 3.10.) was a single band. Additionally, previous haem stains of these samples immediately following column elution showed flavocytochrome c as a single band.

3.3. Discussion.

The method of flavocytochrome c purification according to Morris (1987) yielded 4.0 mg of pure protein from the periplasm prepared from 10 litres of

Plates 3.9. and 3.10. Silver and haem stains of flavocytochrome c purification : method II.

Periplasm was harvested from *S. putrefaciens* grown microaerobically in the presence of fumarate. Flavocytochrome c was purified from this periplasm by precipitation with 60-100% ammonium sulphate which was followed by the sequential application of the resuspended precipitate to DEAE Sepharose and hydroxyapatite. Purity of flavocytochrome c containing fractions was assessed following each purification stage by SDS-PAGE on a 7.5-15% resolving gel followed by silver staining and haem staining. Approximately 10 μg of protein from each fraction was electrophoresed prior to silver staining (Plate 3.9.), and 30 μl of an A_{410} of 0.25 from each fraction electrophoresed before haem staining (Plate 3.10.).

Lane	Plate 3.9.	Lane	Plate 3.10.
1	Fumarate periplasm	1	Horse heart cytochrome-c
2	60-100% ammonium sulphate precipitate	2	Fumarate periplasm
3	DEAE Sepharose fraction	3	60-100% ammonium sulphate precipitate
4	hydroxyapatite fraction	4	DEAE Sepharose fraction
5	Protein standards	5	hydroxyapatite fraction

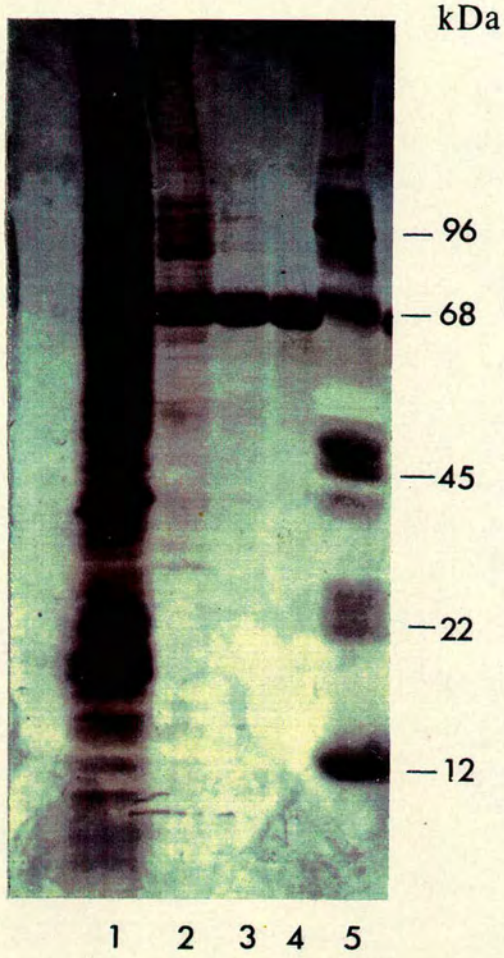


Plate 3.9.

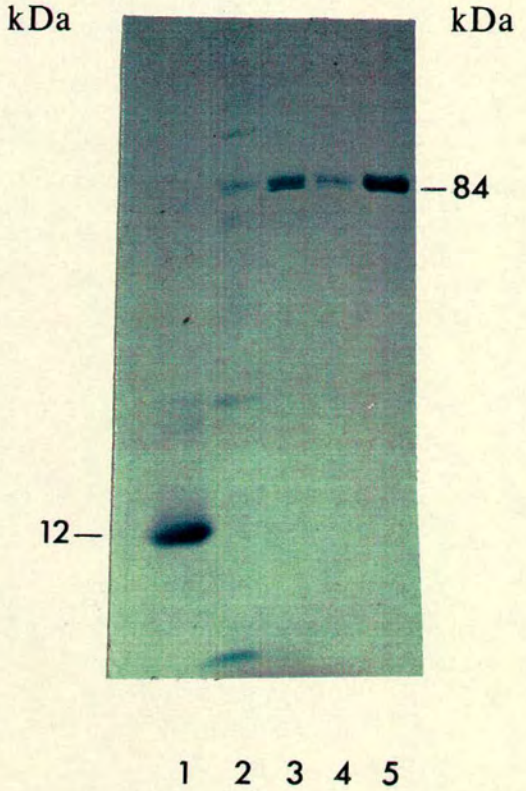


Plate 3.10.

Figure 3.9. Elution profile of the ion exchange flavocytochrome c fraction on hydroxyapatite.

The flavocytochrome c-containing fraction collected following chromatography on DEAE Sepharose was bound to hydroxyapatite equilibrated in T-buffer containing EDTA (5mM). The column was washed with T-buffer (one column volume) then developed by application of an increasing linear gradient of 0-0.5 M K_2HPO_4 in T-buffer. Loading of material, washing, and elution of the sample were carried out at a constant flow rate of 12 ml h^{-1} with 4 ml fractions collected. The A_{280} and A_{410} of each fraction were measured to determine peaks of protein and cytochrome.

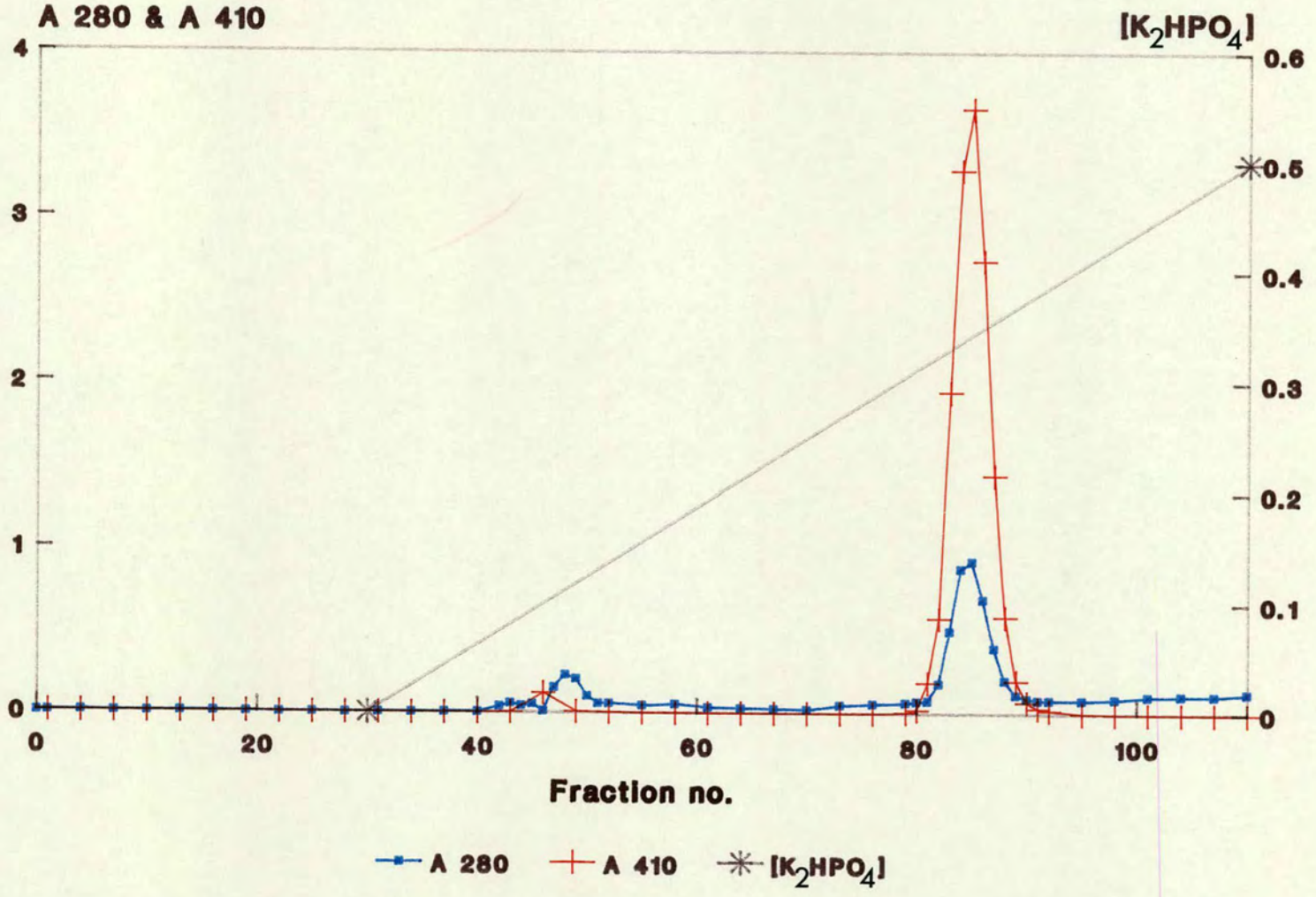

A 280 & A 410
[K2HPO4]
4
3
2
1
0
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
Fraction no.
A 280
A 410
[K2HPO4]

Figure 3.9.

fumarate-grown *S. putrefaciens* (Table 3.1.). Following ion exchange chromatography on DEAE sepharose, the % yield values from A_{410u} (total haem in the fraction, calculated by multiplication of the A_{410} value by the fraction volume) and nmol haem (calculated from the extinction coefficient determined by Morris (1987) for flavocytochrome specific haem) were very similar indicating that all haem in the sample was flavocytochrome specific. This was further confirmed by haem staining of fractions during purification which showed the only cytochrome present following ion exchange chromatography to be flavocytochrome c. Throughout the purification, the ratio of flavocytochrome c specific haem to protein increased as did the purification factor which rose steadily to give a value of 3.08 following chromatography on phenyl sepharose. Following gel filtration, which was the last purification stage and essential for the removal of several minor contaminating proteins, an apparent decrease in purification from 3.08 to 1.87 fold was observed (Table 3.1.). This was surprising as flavocytochrome c was judged to be pure following this stage. It would indicate from these values that gel filtration chromatography was in some way affecting the structure of the protein causing substantial loss of bound haem. However, no haem was detected in fractions other than those contributing to the peak of flavocytochrome which indicated that all haem was associated with flavocytochrome c. Furthermore as the haem groups of c-type cytochromes are covalently bound to the the protein and only released by strong denaturing conditions, it seemed very unlikely that chromatography of flavocytochrome c on Sephacryl S-300 under the described conditions (section 2.6.4.) would result in the release of haem.

The A_{410}:A_{280} ratio was a relatively accurate indicator of flavocytochrome purity following chromatography on DEAE Sepharose which removed all cytochrome with the exception of flavocytochrome c (Table 3.2.).

Table 3.2. Purification of flavocytochrome c : method I.

(A_{410}:A_{280} purity determination)

Flavocytochrome c was purified according to Table 3.2. from the periplasm of *S. putrefaciens* grown microaerobically in the presence of fumarate. Following each purification stage, the absorbances at 280 nm and 410 nm were measured to estimate protein and cytochrome concentration. The absorbance was converted into absorbance units (A_{410U} and A_{280U}) on multiplication with the sample volume. The ratio of cytochrome : protein was used to monitor purity of the sample at each stage.

Purification of flavocytochrome c : method I.

(A_{410}:A_{280} purity determination)

Source	volume (ml)	A_{280}	A_{410}	A_{280U}	A_{410U}	A_{410}:A_{280}
Periplasm	185	4.3	4.5	795	832.5	1.05
Ion Exchange (DEAE Sepharose)	47	2.69	6.8	126.8	320	2.52
Hydroxyapatite	48	0.8	3	38.4	134.4	3.5
Phenyl Sepharose	136	0.115	0.73	26	99.3	3.82
Gel Filtration (Sephacryl S-300)	40	0.4	1.56	16	62.4	3.9

There was no apparent decrease in purity at any stage by this criterion, with the final A_{410}:A_{280} ratio of 3.9 similar to that obtained by Morris (1987) for purified flavocytochrome c. Flavocytochrome c was observed as a single band on silver and haem staining following gel filtration and by these criterion was considered pure. Thus the apparent decrease in purity following the final purification stage of gel filtration was thought to be due to some technical factor, possibly chemical interference in the sample which affected the protein assay giving an inaccurately high value for protein content.

The second purification procedure involved 3 stages following preparation of periplasm: ammonium sulphate fractionation of periplasm, chromatography of the 60-100% precipitate on DEAE sepharose, then finally chromatography of the pooled flavocytochrome c fraction from ion exchange on hydroxyapatite. Flavocytochrome c was pure to homogeneity on haem, silver and Kenacid blue staining of SDS gels following these stages (Plates 3.10 and 3.11).

The yield of protein from the second purification (3.3%) was slightly lower than the first (3.9%). The % yield values obtained for total haem and flavocytochrome specific haem showed that $(NH_4)_2SO_4$ precipitation was effective in removal of most periplasmic cytochromes other than flavocytochrome c and that the haem content in fractions following chromatography on DEAE Sepharose and hydroxyapatite was due only to the flavocytochrome c. There was a decrease in the purification factor following $(NH_4)_2SO_4$ precipitation as estimated by haem content, from 1 to 0.34 further confirming the removal of cytochromes other than flavocytochrome c. In the early purification stages, estimating purity of flavocytochrome c by measuring fumarate reductase activity was considered to be more accurate than haem determination where other cytochromes are present. There is no evidence to

suggest the presence of more than one periplasmic fumarate reductase in *S. putrefaciens*.

The protein content, specific and total fumarate reductase activities of the flavocytochrome-containing fraction were determined at each stage of the second purification (Table 3.3.). A purification of 6.04 fold was obtained yielding 5.96 mg of purified flavocytochrome c and 20% of the total periplasmic fumarate reductase activity. An approximately twofold increase in the specific activity of the enzyme occurred at each stage. The A_{410}:A_{280} ratio increased from 0.85 to 3.89 following the final purification stage (Table 3.4.) which was a purity ratio comparable to that obtained for the previous purification. Purity of flavocytochrome c throughout this purification was also determined from the haem:protein ratio (Table 3.5.). This purification procedure gave a final purification fold of 3.5.

The overall percentage of flavocytochrome c protein in periplasm isolated from microaerophilic cultures, with fumarate as terminal electron acceptor, was calculated as 33% in the first purification and 26% in the second. The differences in the amount of flavocytochrome c purified from the periplasm may not be due only to the different purification procedures, but will also be affected by the stringency of the culture conditions which induce flavocytochrome c, and the effectiveness of the osmotic shock in releasing the flavocytochrome into the periplasm.

Ammonium sulphate was chosen as the first stage of the second purification following preparation of periplasm as it is suitable for protein precipitation, being highly soluble, non-toxic and effective in enzyme stabilisation, in addition to which the high salt concentration during ammonium sulphate fractionation prevents proteolysis and bacterial action (Scopes, 1982). This was advantageous for the initial purification stage as, although no

Table 3.3. Purification of flavocytochrome c : method II.

(fumarate reductase activity determination)

Flavocytochrome c was purified from periplasm prepared from *S. putrefaciens* grown microaerobically in the presence of fumarate according to the procedure in Table 3.2. The protein concentration following each purification stage was determined by the Folin-Ciocalteau protein assay (section 2.8.). The specific[1] and total[2] fumarate reductase activities were measured following each purification stage to evaluate overall yield and purification fold.

Purification of flavocytochrome c : method II.

(Fumarate reductase activity determination)

Source	volume (ml)	Protein (mg ml^{-1})	Specific Activity[1]	Purification (fold)	Total Protein (mg)	Total Activity[2]	Yield (%)
Periplasm	810	0.216	37.1	1	175	6491	100
60-100% $(NH_4)_2SO_4$	34	2.51	58.9	1.59	85.36	5026	77.4
Ion Exchange (DEAE Sepharose)	39	0.437	138	3.72	17.05	2351	36.2
Hydroxyapatite	30	0.199	224	6.04	5.96	1337	20.6

Table 3.4. Purification of flavocytochrome c : method II.

(A_{410}:A_{280} purity determination)

Flavocytochrome c was purified according to Table 3.4. from the periplasm of *S. putrefaciens* grown microaerobically in the presence of fumarate. Following each purification stage, the absorbances at 280 nm and 410 nm were measured to estimate protein and cytochrome concentration. The absorbance was converted into absorbance units (A_{410U} and A_{280U}) on multiplication with the sample volume. The ratio of cytochrome : protein was used to monitor purity of the sample at each stage.

Purification of flavocytochrome c : method II.

(A_{410}:A_{280} purity determination)

Source	volume (ml)	A_{280}	A_{410}	A_{280U}	A_{410U}	A_{410}:A_{280}
Periplasm	810	0.806	0.689	653	558	0.85
60-100% $(NH_4)_2SO_4$	34	3.2	3.5	109	120	1.1
Ion Exchange (DEAE Sepharose)	39	0.774	2.43	30.19	94.6	3.13
Hydroxyapatite	30	0.586	2.19	17.6	65.98	3.89

Table 3.5. Purification of flavocytochrome c : method II.

(cytochrome determination)

Flavocytochrome c was purified from periplasm prepared from *S. putrefaciens* grown microaerobically in the presence of fumarate according to the procedure in Table 3.5. The protein concentration following each purification stage was determined by the Folin-Ciocalteau protein assay (section 2.8.), and haem content was estimated from the absorbance at 410 nm assuming an extinction coefficient of 730 mM^{-1} cm^{-1} for flavocytochrome haem (Morris, 1987).

Purification of flavocytochrome c : method II.

(cytochrome determination)

Source	A_{410u} (% Yield)	Total Protein (%Yield)	nmol haem (%Yield)	nmol haem/mg protein	Purification (Fold)
Periplasm	558 (100)	175 (100)	764 (100)	4.31	1
60-100% $(NH_4)_2SO_4$	120 (21)	85.6 (48)	164 (21.5)	1.9	0.44
Ion Exchange (DEAE Sepharose)	94.6 (17)	17.05 (9.6)	129 (16.8)	7.58	1.76
Hydroxyapatite	65.98 (11.7)	5.96 (3.3)	90 (11.8)	15	3.5

periplasmic proteases have been detected in *S. putrefaciens* NCMB400 (Clark, 1984), some leakage of cytoplasmic contents during the osmotic shock may have released intracellular proteases. Two peptidases have been discovered in the periplasm of Alteromonas B-207 each sensitive to EDTA (Lee & Merkel, 1981., Merkel *et al*, 1980) so the presence of EDTA throughout purification may be effective against endogenous protease activity.

Ammonium sulphate precipitation of protein is dependent on relative hydrophobicity. In an aqueous solution, water molecules aggregate at the hydrophobic regions of the protein. Raising the salt concentration by addition of ammonium sulphate results in free water molecules becoming scarce, which is followed by removal of aggregated water molecules from the protein hydrophobic regions. Thus, the exposed hydrophobic regions of the protein aggregate together and the protein precipitates out of solution (Scopes, 1982). Generally hydrophobic proteins precipitate at low salt concentrations and hydrophilic proteins at higher concentrations, although additional factors such as protein concentration and composition of the sample affect precipitation.

Precipitation of flavocytochrome c in high salt concentration (60-100%) was in agreement with earlier work by Morris (1987) who proposed that it was hydrophilic. As mentioned previously ammonium sulphate precipitation resulted in the loss of the second eluting cytochrome when ion exchange profiles from the two purifications were compared. This cytochrome was previously characterised as being rather hydrophobic (Morris, 1987) which would indicate that it was precipitated at a lower ammonium sulphate concentration.

Ammonium sulphate fractionation had other additional advantages as a first purification stage, the first being concentration of the periplasmic fraction containing flavocytochrome c resulting in easier processing for the next stage, and the second was loss of periplasmic proteins precipitating at saturations of

less than 60% therefore increasing purity of the sample. 83.3% of the total periplasmic fumarate reductase activity was recovered by ammonium sulphate precipitation, 77.6 % of which was located in the 60-100% fraction (Table 3.6) indicating minimal inhibition of enzyme activity by ammonium sulphate.

Subsequent flavocytochrome purifications using ammonium sulphate fractionation of periplasm as an initial stage will require only two fractionation steps: a first of 0-60% to remove unwanted cytochromes and proteins and a second of 60-100% to concentrate the flavocytochrome c-containing fraction.

Ion exchange chromatography on DEAE sepharose was chosen as the next purification stage due to its effective separation of periplasmic proteins (Morris, 1987). The principle of ion exchange chromatography involves the binding of proteins by electrostatic forces to the charged groups on the matrix thereby displacing the counter ions (Cl^-). Proteins are displaced from the matrix on encountering gradually increasing concentrations of counter ion during development of the column which compete for sites on the matrix with bound protein. Proteins with a high affinity to the matrix bind tightly and require a higher cation concentration to facilitate release than protein with a lower affinity. Ion exchange chromatography effectively removed all protein from the fraction containing flavocytochrome c with the exception of several minor co-eluting proteins.

The next purification stage was chosen to complement the preceding stages. The basic mechanism of hydroxyapatite chromatography is different from $(NH_4)_2SO_4$ fractionation and ion exchange chromatography in that the absorption of acidic proteins is believed to be due to the interaction of acidic groups on the protein with calcium sites at the surface of the hydroxyapatite crystals. Elution of the protein is achieved with competing ions for the calcium sites, particularly those with a high affinity for Ca^{2+} such as phosphate, rather

Table 3.6. Ammonium Sulphate Precipitation of Periplasm.

	Specific Activity[1]	Purification (fold)	Total Activity[2]	Yield (%)
Periplasm	37.1	1	6491	100
0-60% precipitate	11.6	0.316	370	5.6
60-100% precipitate	58.9	1.59	5026	77.6
80-100% supernatant	2.8	0.075	98	0.7

The effect of ammonium sulphate on the recovery of periplasmic fumarate reductase activity was determined as above.

1 = μmol MV^{++} oxidised min^{-1} mg $protein^{-1}$.

2 = μmol MV^{++} oxidised min^{-1} x mg protein in sample.

than Cl^- (Gorbunoff, 1984; Gorbunoff & Timasheff, 1984). Following this stage, flavocytochrome c was pure to homogeneity.

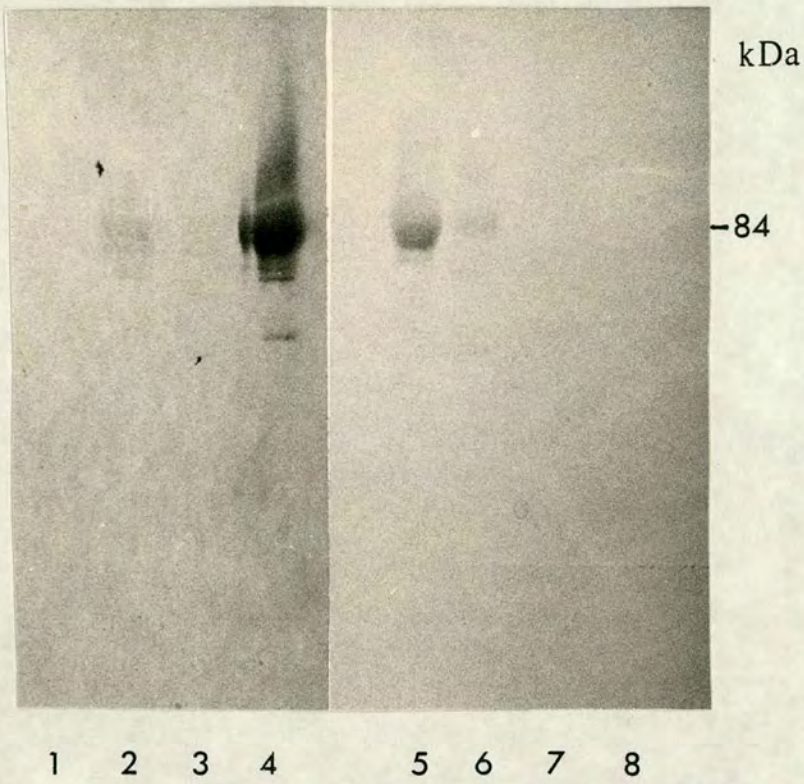
There were two main advantages of this new purification procedure. The first was the reduction in the number of stages involved in the purification, therefore decreasing the time taken to purify the protein. Secondly, the reduced processing of the protein may also have been a contributing factor to the apparent lack of degradation throughout purification, although the presence of EDTA in the elution buffer at all stages of purification also helped prevent proteolysis. EDTA acts as a metal chelator and as even trace levels of heavy metals can substantially affect enzyme activity (Scopes, 1982), the ability of EDTA to bind these will maintain enzyme activity of proteins sensitive to heavy metals.

3.4. Specificity of antibody to flavocytochrome c.

Antibody was raised to purified flavocytochrome c and the sheep blood processed to yield purified IgG (section 2.9.). The specificity of antibody raised to flavocytochrome c was determined by Western blotting against purified flavocytochrome, and also against total cell lysates of aerobically and microaerobically grown *S. putrefaciens* and *E. coli* MM294 (Plate 3.11). This blot showed that antibody bound specifically to flavocytochrome c in both purified form and in microaerobic cell extracts from *S. putrefaciens* (cultured separately with fumarate and TMAO as terminal electron acceptors and also with no exogenous electron acceptor).

Other bands of lower molecular weight than flavocytochrome c also bound antibody. These could have been due to minor undetected contaminating proteins present in the original purified flavocytochrome c sample against which antibody was raised. Alternatively, antibody could have been binding to

Plate 3.11. Specificity of antibody raised to purified flavocytochrome c.



Specificity of antibody raised to purified flavocytochrome c was determined by Western blotting with total cell lysates (30 μg) of *S. putrefaciens* and *E. coli* MM294 cultured by various growth conditions.

Lane

1 Mid-exponential *S. putrefaciens*.

2 Microaerobic *S. putrefaciens* with fumarate as terminal electron acceptor.

3 Microaerobic *S. putrefaciens* with TMAO as terminal electron acceptor.

4 Purified flavocytochrome c (2 μg).

5 Purified flavocytochrome c (2 μg).

6 Microaerobic *S. putrefaciens*.

7 Mid-exponential *E. coli* MM294.

8 Microaerobic *E. coli* MM294.

proteolytic products of flavocytochrome c. The smaller molecular weight bands developed more slowly than flavocytochrome c indicating that they were either less specific for the antibody, or represented a substantially smaller proportion of the original protein sample. In either case, there was no apparent binding of the antibody to any other protein from aerobically cultured *S. putrefaciens* or to *E. coli* cultured either aerobically or under oxygen limited conditions. This indicated that this antibody preparation was specific for and had the ability to detect flavocytochrome c against a background of other proteins.

The ability of IgG raised against flavocytochrome c to precipitate fumarate reductase activity from periplasmic extracts of *S. putrefaciens* and purified flavocytochrome c was investigated. A protein concentration corresponding to a fumarate reductase activity of 40 - 45 μmol MV^{++} oxidised $min^{-1}ml^{-1}$ was used in each sample as at this activity even very slight enzyme inhibition was easily measured. The inhibition curves obtained by measuring fumarate reductase activity of the sample following preincubation with IgG are presented in Figures 3.10 and 3.11 . A two-step inhibition was observed for both periplasm and purified protein: the first stage showed a rapid reduction of fumarate reductase activity on preincubation with a relatively small volume (1-20 μl) of flavocytochrome specific IgG, followed by a much slower secondary loss of remaining activity on preincubation with larger volumes (up to 400 μl) of IgG. The inhibition was less rapid with purified flavocytochrome c than periplasm. This was thought to be due to the slightly higher fumarate reductase activity of purified flavocytochrome c in the assay compared to periplasmic activity, thus requiring a proportionally greater concentration of antibody to inhibit enzyme activity to the same extent. The antibody was thought not to be binding to a region involved directly in fumarate reduction, such as the enzyme-substrate binding site, or the flavin prosthetic group which is required for

Figure 3.10. Titration of periplasmic fumarate reductase activity with flavocytochrome c specific IgG.

A sample of periplasm which gave a fumarate reductase activity of 40 μ mol MV^{++} oxidised min^{-1} was preincubated with IgG raised to purified flavocytochrome c for 10 min at room temperature prior to assay for fumarate reductase activity.

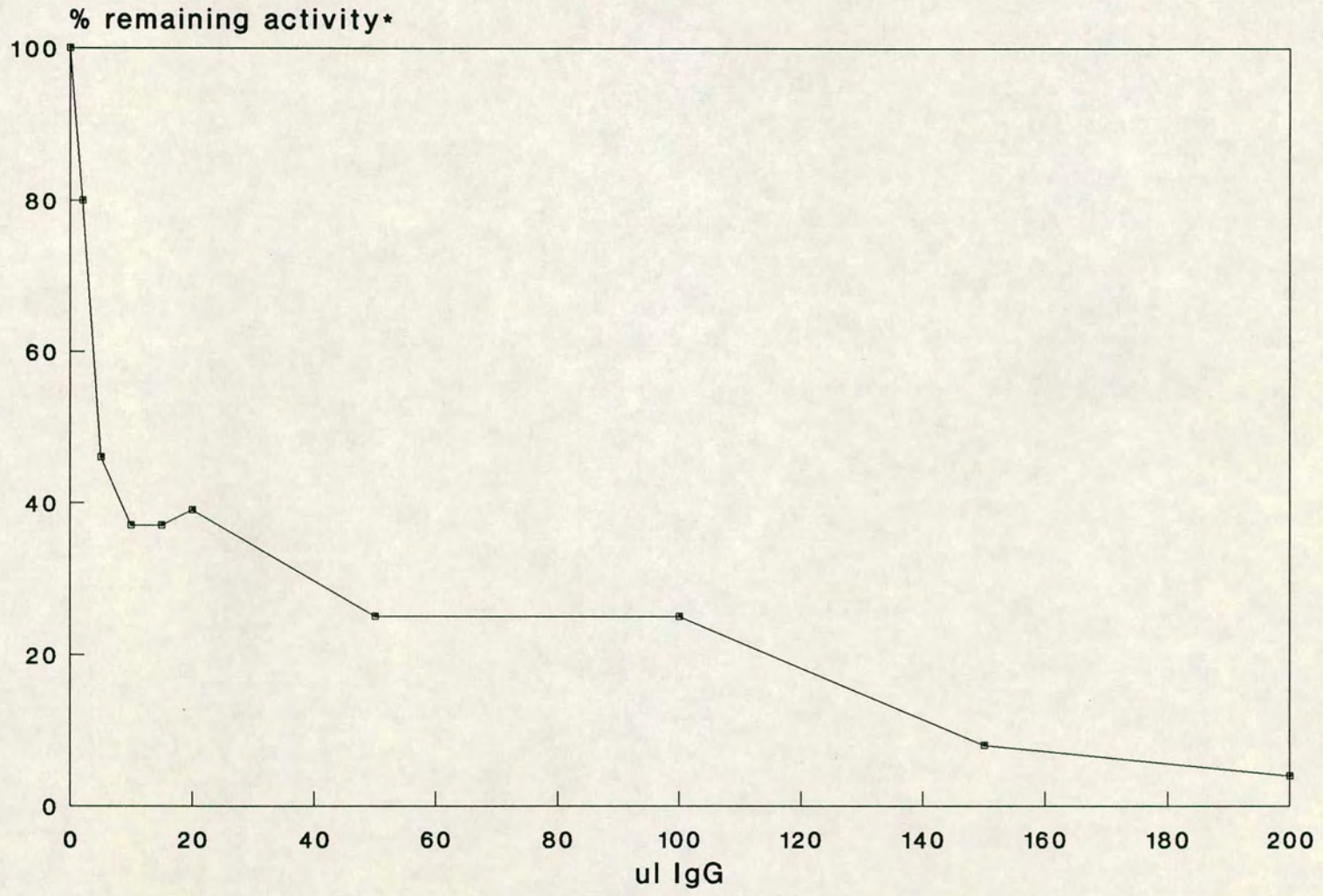

% remaining activity*
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
ul IgG

Figure 3.10.

Figure 3.11. Titration of fumarate reductase activity from purified flavocytochrome c with flavocytochrome c specific IgG.

A flavocytochrome c concentration corresponding to a fumarate reductase activity of 45 μ mol MV^{++} oxidised min^{-1} was preincubated with IgG raised to purified flavocytochrome c for 10 min at room temperature prior to assay for fumarate reductase activity.

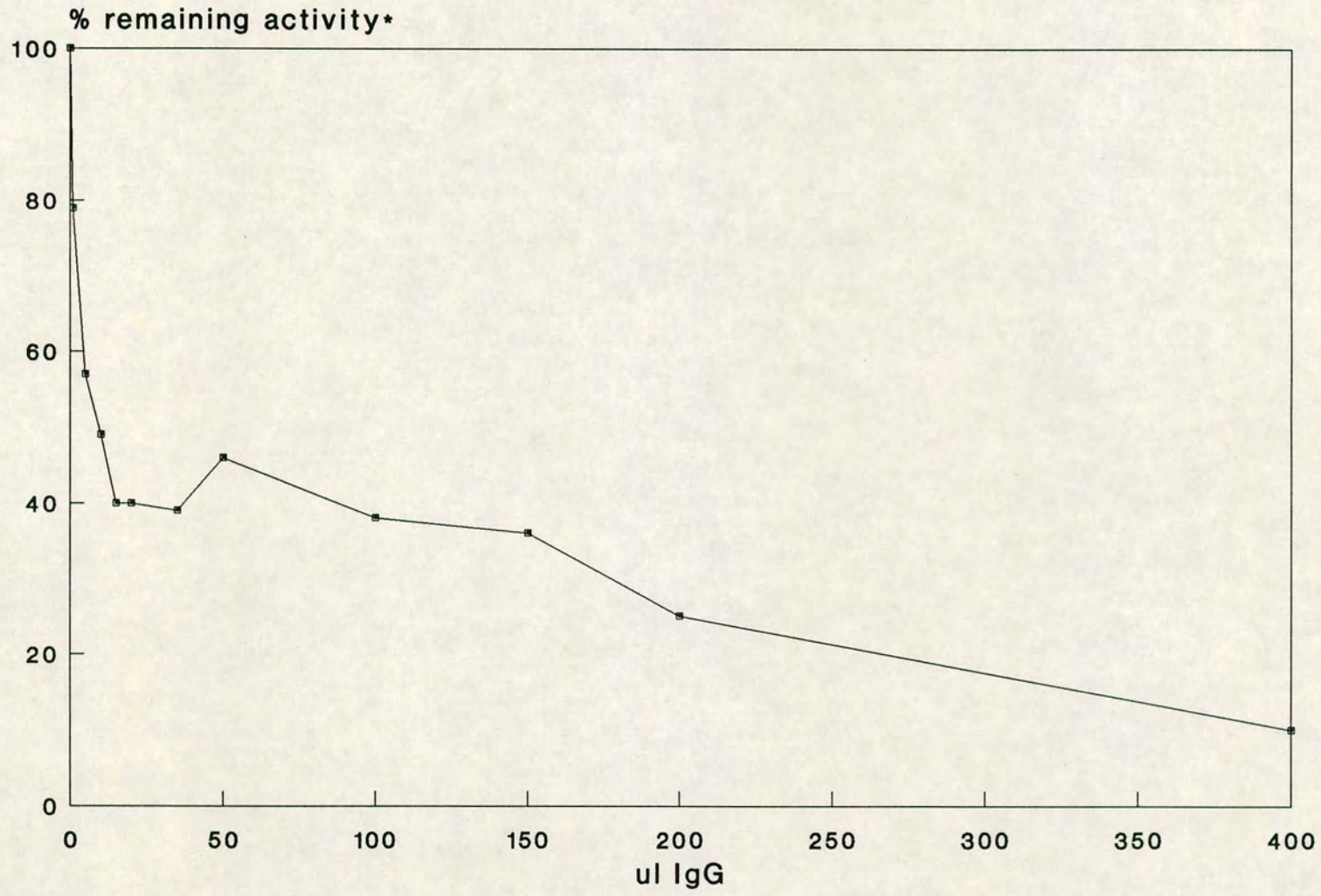

% remaining activity*
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
ul IgG

Figure 3.11.

fumarate reductase activity in other prokaryotes, as this would be expected to result in rapid and complete inhibition of enzyme activity. Instead, the two-step inhibition suggested a less direct type of inhibition perhaps involving conformational flexibility of the protein. At least two species of antibody seemed to be involved in this, one which was specific and reduced enzyme activity to 40% on preincubation with relatively small volumes of IgG, and a second less specific species which resulted in total loss of activity with larger volumes of IgG.

The factors determining immunogenicity of native proteins have been studied with respect to protein structure. Initially it was thought (Sela *et al*, 1967), that the immunogenicity of a protein was contributed to by both continuous regions (stretches of sequential residues) and discontinuous regions (residues from different parts of the sequence brought together by folding of the protein). It was subsequently proposed that the protein determinants most likely to elicit an immune response were discontinuous (Benjamin *et al*, 1984). Thus antibody will recognise both sequence and conformation of the protein with the most immunogenic regions on the protein surface. Antiserum raised to a pure native protein will therefore contain a mixture of low and high affinity antibody to various regions on the protein surface. The exposure of protein to denaturing conditions such as SDS PAGE prior to Western blotting will result in the destruction of the secondary protein structure; however, binding of antibody raised to native protein will still occur to the stretches of amino acids which contributed to the discontinuous epitopes (Barlow *et al*, 1986).

3.5. Fumarate reductase activity of flavocytochrome c.

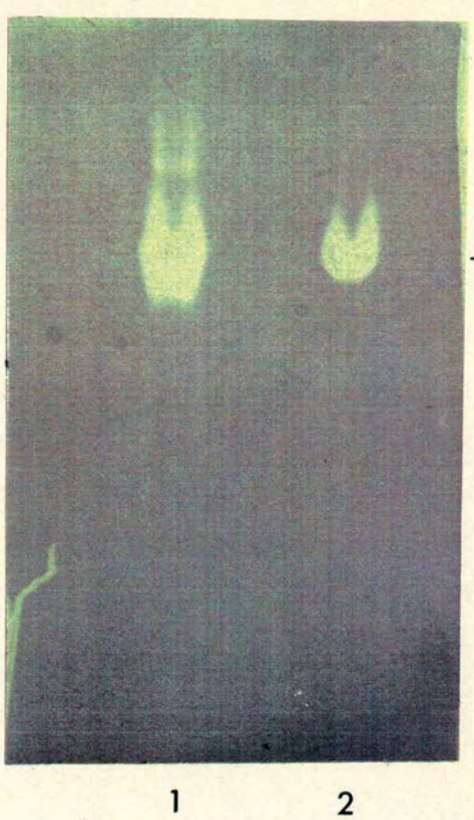
Preliminary experiments to determine the cellular location of the fumarate reductase of *S. putrefaciens* showed that fumarate reductase activity

co-purified at all stages with flavocytochrome c (Ward *et al*, 1987 unpublished results). Furthermore sub-fractionation studies had shown that fumarate reductase activity was periplasmic.

Zymogram staining of purified flavocytochrome c showed a single band of fumarate reductase activity, following native gel electrophoresis (Plate 3.12.). An additional band of greater molecular weight than flavocytochrome c was observed following zymogram staining of periplasmic extracts (from *S. putrefaciens* cultured microaerophilically with fumarate as terminal electron acceptor). This suggested either the presence of two periplasmic fumarate reductases, or a second form of flavocytochrome c. In an attempt to resolve this, periplasm was Western blotted, then developed with IgG raised to purified flavocytochrome c (Plate 3.13). A positive signal to both bands was observed, indicating that both contained epitopes of flavocytochrome c. Thus it was considered that the larger band was flavocytochrome c not fully denatured by electrophoresis under these conditions. As non-covalently bound FAD necessary for enzyme activity is removed by denaturing conditions (Muratsubaki and Katsume, 1982), it was very surprising that flavocytochrome c still retained fumarate reductase activity following SDS-PAGE, although activity was very much reduced with only a very faint band observed (and for this reason, the results are not presented). This indicated some partially active native structure remaining after treatment with SDS and mercaptoethanol, or partial renaturation of the protein in zymogram buffer prior to assay for fumarate reductase activity. This observation will have to be investigated in detail by enzyme assay for fumarate reductase activity following denaturation of purified flavocytochrome c.

The possibility that flavocytochrome c acted physiologically as a fumarate reductase was then investigated in more detail.

Plate 3.12. Zymogram of periplasm and purified flavocytochrome c.



Periplasm (approximately 10 μg) from *S. putrefaciens* grown microaerobically in the presence of fumarate, and purified flavocytochrome c (2 μg) were electrophoresed on a 10% native gel. Zymogram buffer (100 mM K_2HPO_4, 100 mM NaCl, 1 mM $MgSO_4$ 0.1 mM methyl viologen; pH 7.4) was degassed by continual sparging with oxygen-free argon, then methyl viologen reduced by addition of sodium dithionite (2% in 10 mM NaOH). The gel was immersed in this solution for approximately 20 min before addition of fumarate (pH 7) to 1 mM.

Lane	
1	Periplasm
2	Purified flavocytochrome c

Plate 3.13. Western blot of periplasm from fumarate-grown *S. putrefaciens.*

Approximately 30 μg of periplasm isolated from *S. putrefaciens* grown microaerobically in the presence of fumarate, and 2 μg purified flavocytochrome c were transferred to nylon membrane following native PAGE (7.5-15% resolving gel) then developed with IgG raised to purified flavocytochrome c.

Lane

1 periplasm

2 purified flavocytochrome c

3.6. Kinetic analysis of flavocytochrome c

To determine the specificity of flavocytochrome c for fumarate and succinate, the fumarate reductase and succinate dehydrogenase activities of flavocytochrome c were measured with varying substrate concentration. Enzyme activity was measured at 20°C in buffer at the optimum pH. No inhibition of enzyme activity was detected at saturating substrate concentrations. The rates of enzyme activity at each substrate concentration were plotted on to Lineweaver-Burk plots (Figs. 3.12 and 3.13). The half maximal substrate concentration (K_M) and the maximum rate of enzyme reaction (V_{max}) were determined from the intercept on the ordinate and abscissa respectively, from the line plotted by linear regression analysis of the values obtained for each substrate concentration. Correlation coefficients of 0.99 and 0.96 were calculated for fumarate and succinate repectively. The K_M, V_{max} and kcat (a measure of turnover, defined as the no. of molecules of substrate converted to product per second and calculated from the rate of activity at the lowest saturating enzyme concentration) values for fumarate and succinate are presented in Table 3.7.

The K_M, V_{max} and kcat values for fumarate compared to those for succinate indicated that flavocytochrome c had a greater specificity for fumarate than succinate due to the following observations:

1) The K_M for fumarate was 20-fold less than that for succinate indicating a greater specificity of flavocytochrome c for fumarate than succinate.

2) The kcat for fumarate reduction was approximately 3000 fold greater than that for succinate oxidation showing a faster rate of conversion from fumarate to succinate than vice versa.

However, the Lineweaver-Burk plot preferentially weights values derived from low substrate concentrations. This was of particular concern in the plot

Figure 3.12. Lineweaver-Burk plot of flavocytochrome c with fumarate as substrate.

The reciprocal molar substrate concentration 1/S was plotted against the reciprocal enzyme rate 1/V (μmol MV^{++} oxidised min^{-1} mg $protein^{-1}$).

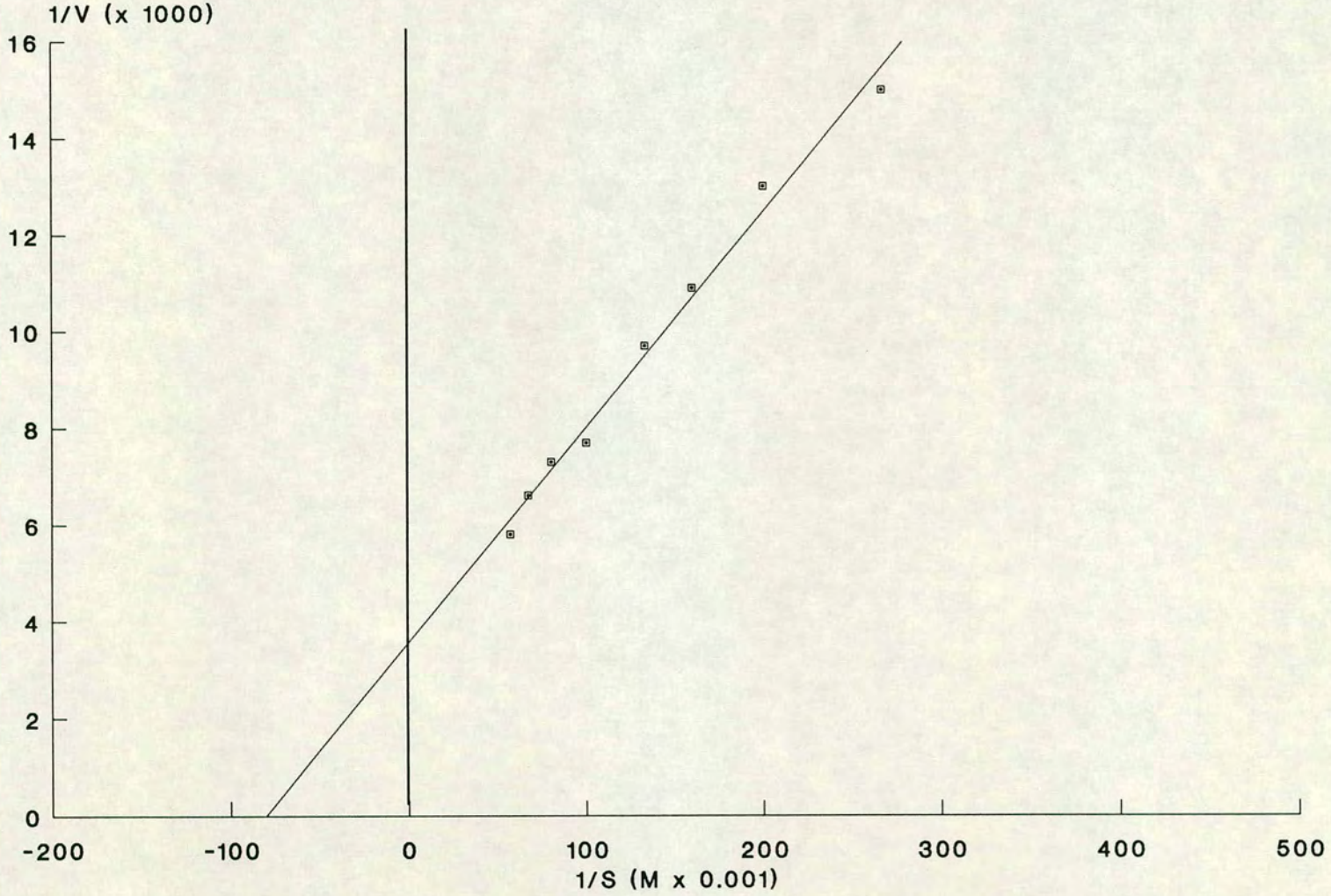

1/V (x 1000)
16
14
12
10
8
6
4
2
0
-200
-100
0
100
200
300
400
500
1/S (M x 0.001)

Figure 3.13. Lineweaver-Burk plot of flavocytochrome c with succinate as substrate.

The reciprocal molar substrate concentration 1/S was plotted against enzyme rate 1/V (μmol DCPIP reduced min^{-1} mg $protein^{-1}$).

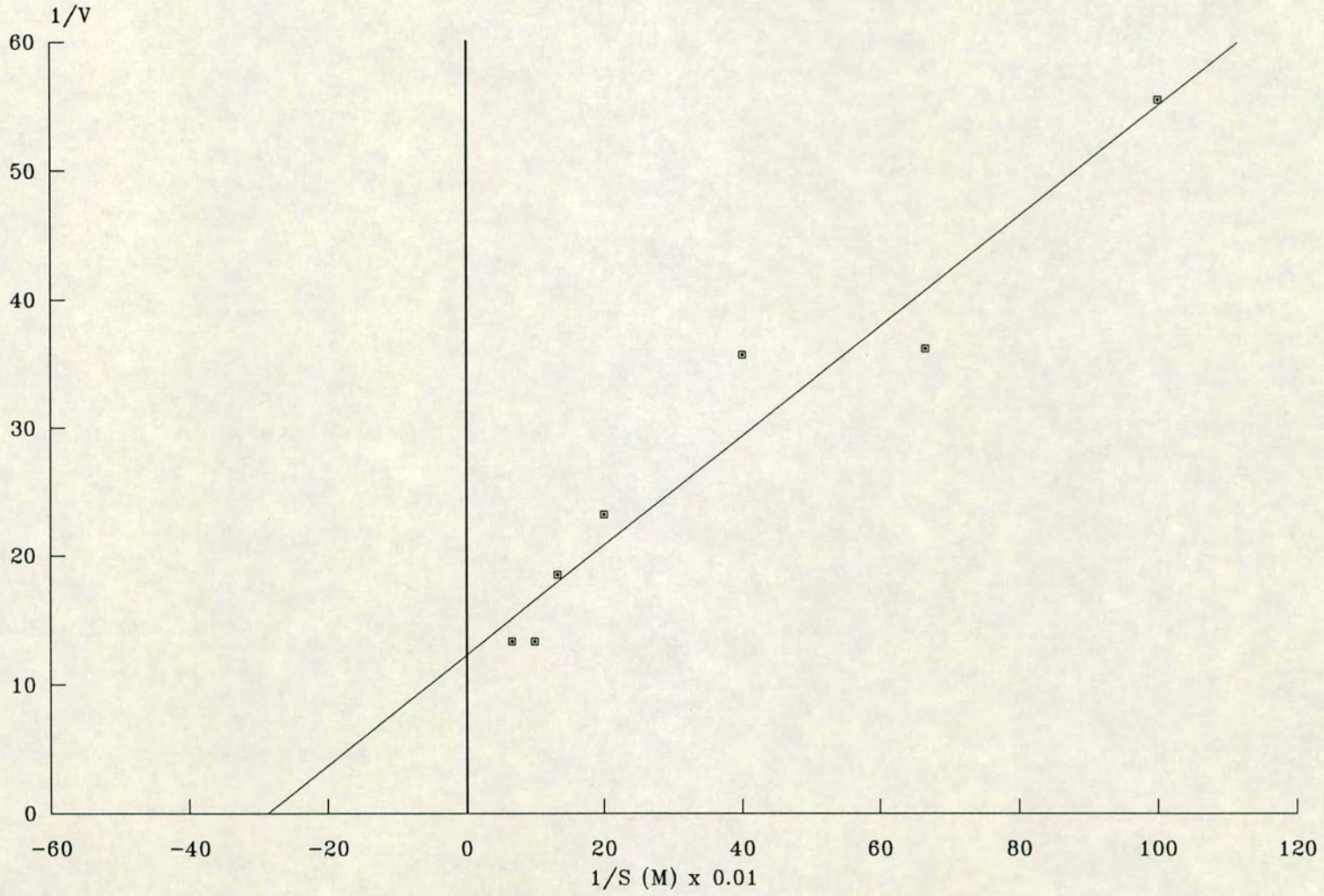

1/V
60
50
40
30
20
10
0
-60
-40
-20
0
20
40
60
80
100
120
1/S (M) x 0.01

Table 3.7. Kinetic Properties of flavocytochrome c.

		Fumarate	Succinate
K_M	(μM)	12.6	356
Vmax	(μmol/min/ mg protein)	278	0.08
kcat	(s^{-1})	287	0.086

Values were calculated from the Lineweaver-Burk plots.

with succinate as substrate which showed substantial scattering of points. The rates of activity at different substrate levels were subsequently analysed by the non-linear regression analysis method of Gamp *et al* (1980), which gives an equal weighting to each value. These results are presented in Table 3.8., Figure 3.14. and Figure 3.15.. The K_M fumarate calculated by this method was almost identical to that derived from the Lineweaver-Burk plot of 12.6 μM, and the Kobs. values closely correlated to those of Kcalc (Figure 3.14.). This indicated that the original experimental data were relatively accurate. The K_M succinate of 630 μM obtained from non-linear regression analysis was much higher than that derived from the Lineweaver-Burk plot (356 μM), and the Kobs. values were scattered on the Kcalc. curve, further indicating that these data were not as accurate as that for fumarate reduction. These results confirmed that flavocytochrome c was highly specific for fumarate, and much less for succinate.

The fumarate reductase enzyme has been characterised in other bacteria, in particular *E. coli* and *V. succinogenes* where the Km of the purified enzyme for the substrates fumarate and succinate has been determined. The fumarate reductase from *E. coli* had Km values of 17 μM and 1000 μM for fumarate and succinate respectively (Hirsch *et al*, 1963; Dickie & Weiner, 1979), being similar to flavocytochrome c in it's high specificity for fumarate. The *V. succinogenes* fumarate reductase was less specific for both fumarate and succinate with K_M values of 350 μM and 2000 μM repectively (Unden *et al*, 1980).

The enzymes from *E. coli* and *V. succinogenes* differ from flavocytochrome c in both their cellular location and subunit structure. As previously discussed in the Chapter 1, flavocytochrome c seems to be composed of a single subunit, in contrast to the fumarate reductases of *E. coli* and *V. succinogenes* which are composed of four subunits in *E. coli* (Dickie & Weiner, 1979; Lemire *et al*, 1982) and three subunits in *V. succinogenes*, and bound to the

Table 3.8. Flavocytochrome c K_M evaluation by non-linear regression analysis.

[Fumarate] M x 10^5	Kobs. x 10^5	Kcalc. x 10^5
1.75	0.136	0.133
1.5	0.121	0.123
1.25	0.109	0.112
1.0	0.103	0.099
0.75	0.082	0.083
0.65	0.073	0.074
0.5	0.062	0.063
0.375	0.052	0.05

K_M fumarate = 14 μM +/- 1.4

[S] M = 2.35×10^{-5}

[Succinate] M x 10^3	Kobs. x 10^3	Kcalc. x 10^3
1.5	7.5	7.65
1.0	7.5	6.66
0.75	5.4	5.9
0.5	4.3	4.81
0.25	2.8	3.08
0.15	2.76	2.09
0.1	1.80	1.49

K_M succinate = 630 μM +/- 160

[S] M = 1×10^{-3}

The K_M values presented in this table were derived from the non-linear regression analysis computer programme of Gamp *et al*, (1980), which measures enzyme activity (Kcalc.) at a particular substrate concentration. These rates of activity are derived from the lowest saturating substrate concentration giving the maximum rate of enzyme activity (S) and the predicted K_M for that particular substrate (see Table 3.7.). Experimental data Kobs. (used for the Lineweaver-Burk plots in Figures 3.12. and 3.13.) is listed above with the computer derived values at identical substrate concentrations. Kcalc. and Kobs. are expressed as μmol MV^{++} oxidised min^{-1} for fumarate and μmol DCPIP reduced min^{-1} for succinate. The Kobs. and Kcalc. data for fumarate and succinate are compared graphically in Figures 3.14 and 3.15.

Figure 3.14. Predicted curve of flavocytochrome c fumarate reductase activity.

Values of V calculated were obtained by the non-linear regression analysis computer programme of Gamp *et al* (1980) from experimental flavocytochrome c fumarate reductase activity (V observed)

where V = μmol MV^{++} oxidised min^{-1}.

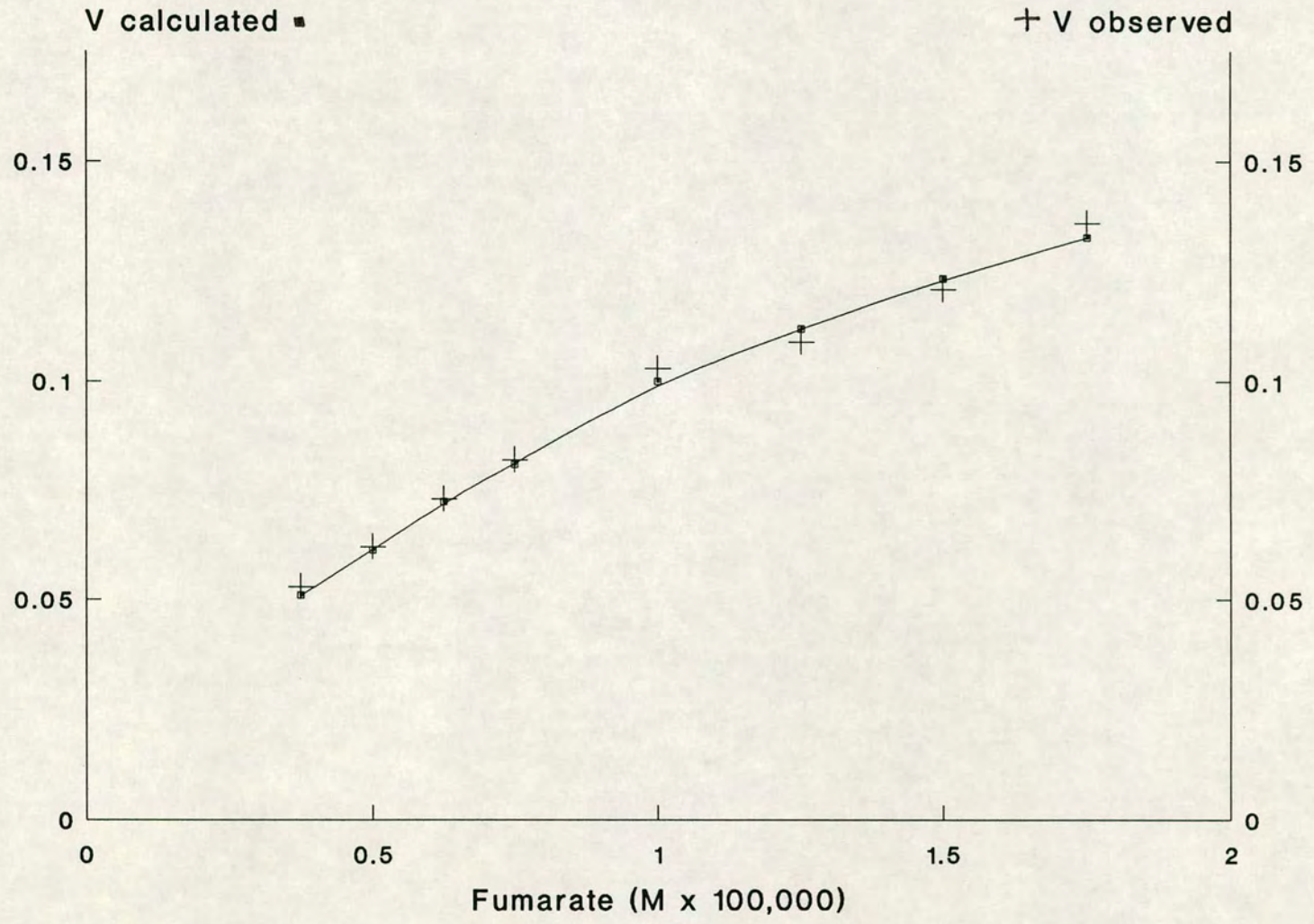
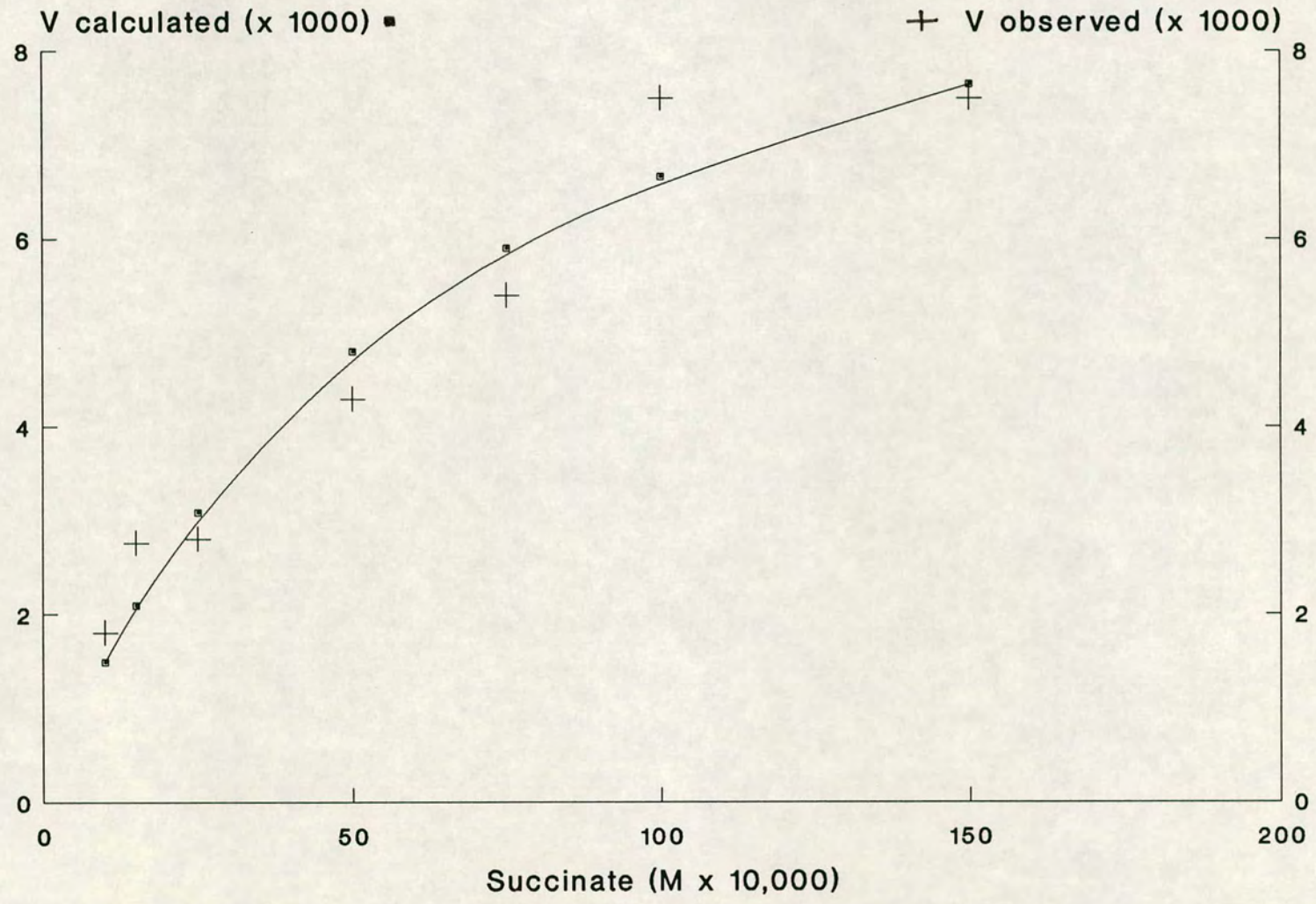

V calculated ▪
+ V observed
0.15
0.1
0.05
0
0.15
0.1
0.05
0
0
0.5
1
1.5
2
Fumarate (M x 100,000)

Figure 3.15. Predicted curve of flavocytochrome c succinate dehydrogenase activity.

Values of V calculated were predicted by the non-linear regression analysis computer programme of Gamp *et al* (1980) from experimental flavocytochrome c succinate dehydrogenase activity data (V observed)

where V = μmol DCPIP reduced min^{-1}.


V calculated (x 1000) ■
+ V observed (x 1000)
8
6
4
2
0
0
50
100
150
200
Succinate (M x 10,000)

inner aspect of the cytoplasmic membrane by hydrophobic anchor proteins. Furthermore, the flavin moiety of flavocytochrome c is non-covalently bound FAD; that of both fumarate reductases from *E. coli* (Dickie & Weiner, 1979) and *V. succinogenes* (Kroger & Innerhoffer, 1976) is covalently bound FAD. It was considered, therefore, that the only similarity between flavocytochrome c and the other enzymes was the associated fumarate reductase and succinate dehydrogenase activities.

Anaerobically induced soluble fumarate reductases with non-covalently bound FAD have been isolated from other organisms such as baker's yeast (Muratsubaki and Katsume, 1982) and *D. multispirans* (He *et al*, 1986). Both have a K_M for fumarate of approximately 0.2 M so are very much less specific for fumarate than flavocytochrome c. Both are cytoplasmic with the enzyme from baker's yeast being a single subunit of 58.8 kDa and that of *D. multispirans* composed of 4 subunits of native molecular weight 132 kDa and attached to the inner aspect of the cytoplasmic membrane by anchor proteins. In contrast to flavocytochrome c neither have succinate oxidising capacity. The lack of this activity, at least in baker's yeast, is thought to provide an accumulation of succinate during oxygen limiting conditions.

CHAPTER 4

CLONING AND SEQUENCING FLAVOCYTOCHROME C

4.1. Introduction

Initial attempts to clone the flavocytochrome c gene were based on complementation of an *frdA* mutation in *E. coli* (Chapter 5). An alternative strategy to clone the flavocytochrome c gene was by using an expression vector system. Expression libraries have been extensively used for cloning eukaryotic genes (see Chapter 1) and should work similarly for the cloning of bacterial genes. It was decided to construct a library in the pEX series of expression vectors, which direct expression of cloned DNA as *lacZ* fusions as the expression of cloned inserts in all three reading frames maximises the chances of identifying a particular product. Furthermore, the presence of the polylinker cloning site at the 3' end of the truncated *lacZ* gene directs the synthesis of more stable hybrid proteins (Stanley, 1984).

4.2. Preparation of chromosome for cloning

4.2.1. Isolation of chromosomal DNA from *S. putrefaciens*

Chromosomal DNA was prepared from *S. putrefaciens* by the modified protocol according to Maniatis *et al* (1989) (section 2.21.), which was originally developed for *E. coli*. At the stage following addition of lysozyme it was found that incubation of the suspension at 30°C achieved more complete lysis than at room temperature. Subsequently, on addition of proteinase K at 50°C, the combination of a more dilute suspension and good cell lysis facilitated efficient proteolysis which was indicated by a colour change of the suspension from a deep red to pale straw yellow colour. Approximately 500 μg of chromosomal DNA was routinely isolated by this method from a 100 ml overnight culture of *S. putrefaciens*. This was estimated from the A_{260}:A_{280} ratio (section 2.21.) to have a value of 2.0, indicating purity of the sample (uncontaminated with

cellular protein). To ensure that no nuclease activity or extensive chromosomal shearing had occurred during isolation, a sample was electrophoresed in 0.5% agarose. The DNA was observed to migrate as a single band of approximately 20 kb in accordance with pure chromosomal DNA.

4.2.2. Partial digestion of *S. putrefaciens* chromosomal DNA.

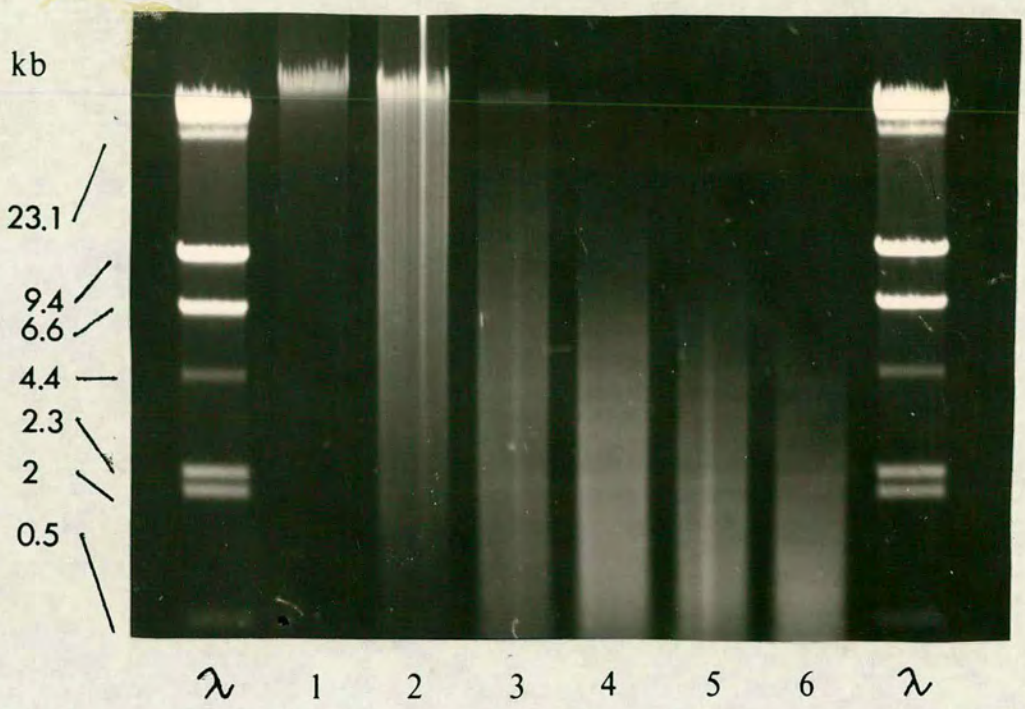
Chromosomal DNA from *S. putrefaciens*, prepared according to section 4.2.1., was partially digested with *Sau3A* to yield suitable fragments for library construction. Preliminary partial digests were performed as described in section 2.22.1. to estimate the digest time which yielded the maximum quantity of DNA fragments of 1-10 kb. From this preliminary experiment (Plate 4.1.) it was proposed that the digest time yielding the majority of fragments of this size was between 5 and 10 minutes following addition of enzyme. Continuing incubation for more than 10 minutes yielded an abundance of fragments which were smaller than 4 kb.

To yield a sufficient quantity of partially digested chromosome, 200 μg of DNA was digested with *Sau*3A for 8 minutes in an appropriately scaled up reaction (section 2.22.2.). The success of this partial digest was determined by electrophoresis of an aliquot on agarose (Plate 4.2.), from which it was observed that suitable partial digestion was obtained yielding a range of fragments from less than 0.5 kb to approximately 20 kb, with the greatest abundance of DNA occurring in the range of 2-10 kb.

4.2.3. Size fractionation of partially digested DNA.

To isolate and concentrate fragments of 1-10 kb from the scaled up partial digest, DNA was size fractionated on 10-40% sucrose gradients. The distribution of DNA and fragment size in the gradient fractions were

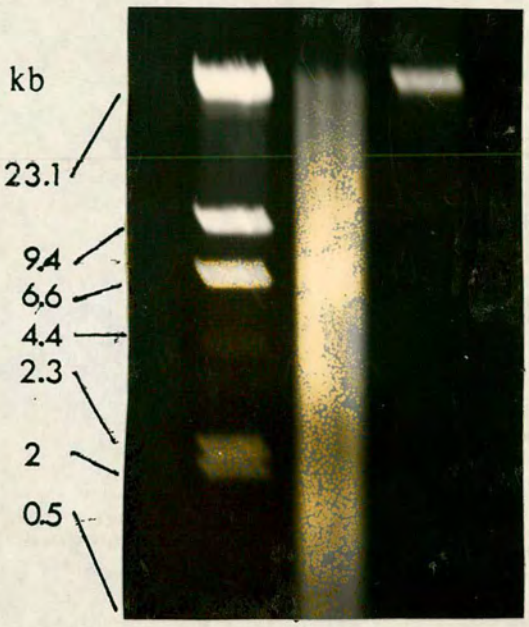
Plate 4.1. Preliminary partial digest of *S. putrefaciens* genomic DNA.



*Sau*3A (3 units) was mixed with genomic DNA (6 μg) preheated in a 37°C water bath. Aliquots containing approximately 1 μg DNA were removed at 0, 5, 10, 13, 16, and 20 intervals following addition of enzyme and the reaction stopped immediately by EDTA, pH 8.0 (final concentration 20 mM). The extent of digestion in each sample was determined by electrophoresis on 0.5% agarose.

Lane	Digest time (min) following addition of *Sau* 3A.
1	0
2	5
3	10
4	13
5	16
6	20

Plate 4.2. Large scale partial digest of *S. putrefaciens* genomic DNA.



Chromosome (200 μg) was digested with *Sau*3A (100 units) for 8 min in a 37°C water bath before inactivation of the enzyme by extraction with phenol/chloroform (1:1). Following ethanol precipitation, the digest was assessed by electrophoresis of approximately 5 μg DNA on 0.5% agarose.

Lane

1	*Sau*3A partial digest (5 μg).
2	Undigested chromosomal DNA (1 μg).

determined by electrophoresis (Plate 4.3.) from which it was observed that the majority of DNA was collected in fractions 12-20. Fractions 1-12 and 22-30 were discarded as containing DNA fragments of more than 10 kb or less than 1 kb respectively. Fractions 13-16 and 17-20 inclusive were pooled separately to yield two samples of size fractionated DNA with fragment sizes ranging from 4-10 kb and 0.5-4 kb, respectively.

4.3. Library construction and screening

4.3.1. Genomic library construction in pEX3.

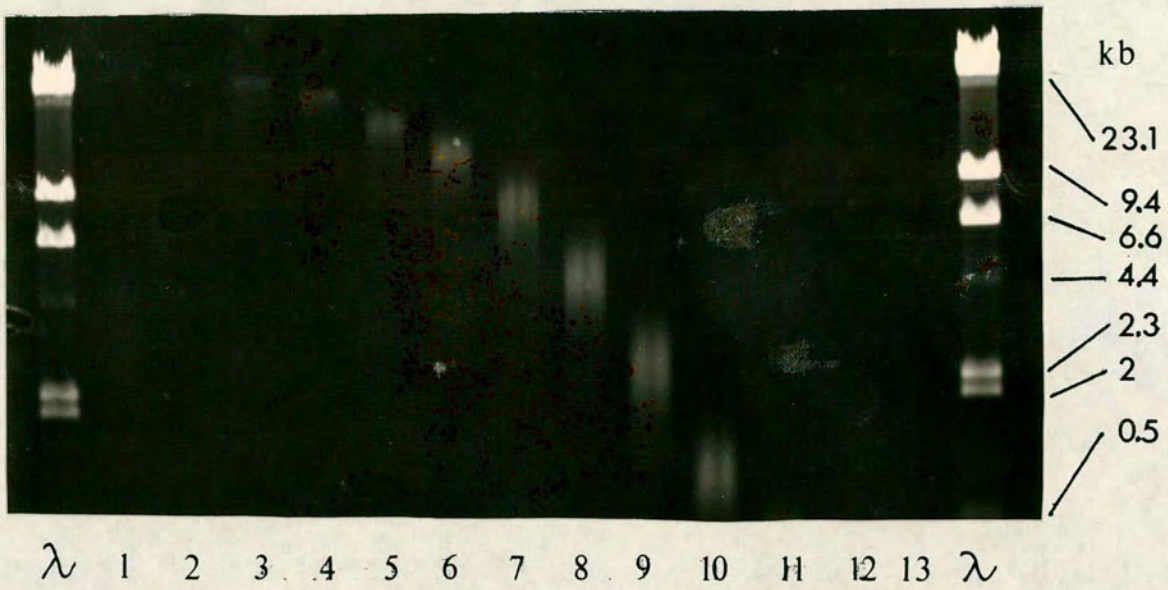
The library was constructed in *E. coli* MM294 containing the plasmid pcI857 (Figure 2.1.) as described in section 2.24. by the ligation of 0.5-4kb *Sau*3A genomic fragments of *S. putrefaciens* into the *Bam*H1 site of pEX3 (Figure 4.1.). A library of 25 000 colonies was obtained, 90% of which were recombinants (assessed by isolating and sizing plasmid from ten independent transformants in comparison to the parent vector).

4.3.2. Library screening.

Although antibody raised to purified flavocytochrome was previously shown to detect flavocytochrome from cell extracts of *S. putrefaciens*, screening of an expression library would require the antibody to have a relatively high affinity for the flavocytochrome. Affinity of the antibody to purified flavocytochrome was assessed (section 2.12.3.) and is described below.

Ten-fold dilutions of the flavocytochrome from 100 μg to 0.1 ng were spotted on to nylon filters which were then treated as for Western blots, except that each of the filters was incubated with a different volume of flavocytochrome specific IgG from 10-500 μl in a volume of 15 ml. Following development of the

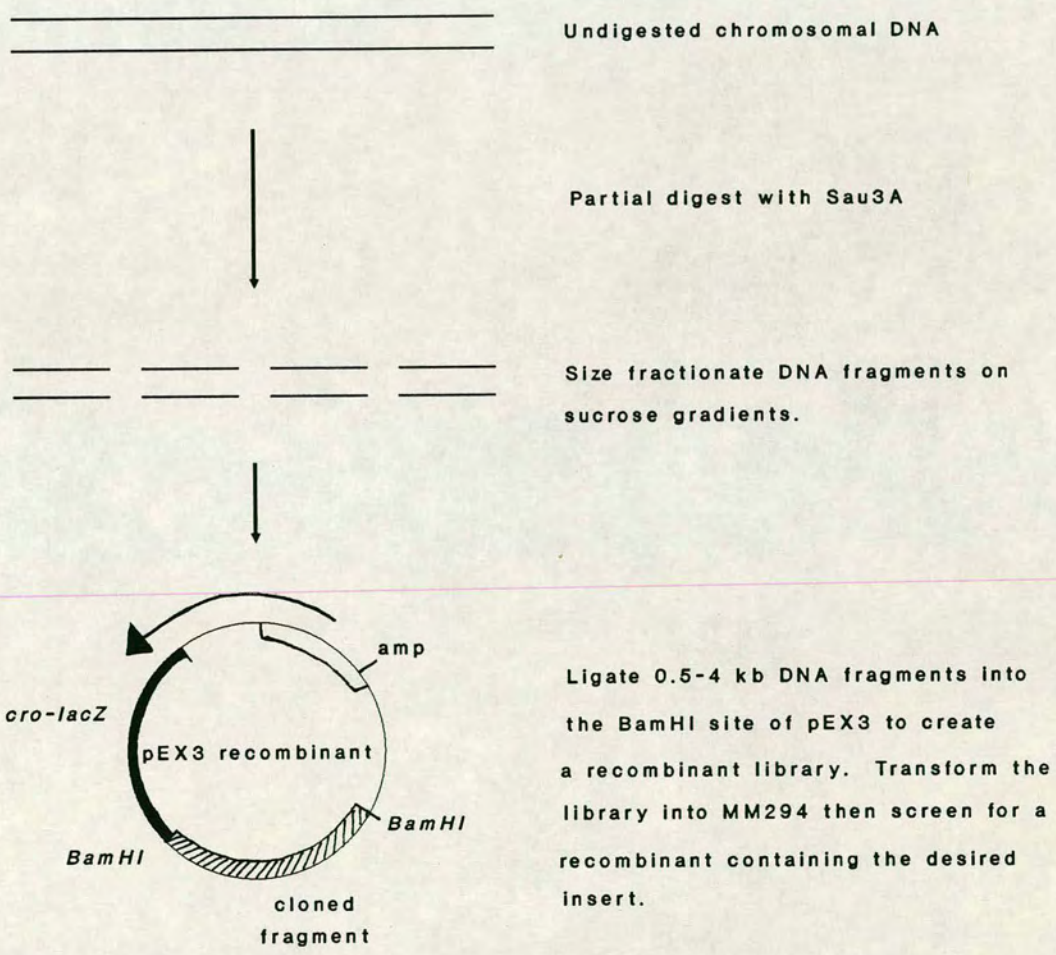
Plate 4.3. Size fractionation of partial digest.



Partially digested chromosome was size fractionated by centrifugation on 10-40% sucrose gradients. Aliquots (0.5 ml) were collected and 20 µl of each alternate fraction electrophoresed on 0.5% agarose. The markers were diluted 1:1 with 40% sucrose to compensate for the sucrose content of the DNA fractions.

Lane	Fraction	Lane	Fraction
1	3	**8**	16
2	4	**9**	18
3	6	**10**	20
4	8	**11**	22
5	10	**12**	24
6	12	**13**	26
7	14		

Figure 4.1. Summary of *S. putrefaciens* genomic library construction in pEX3.



filters it was found that incubation with 100 µl IgG could detect 10 ng and possibly as little as 1 ng of flavocytochrome c (Plate 4.4.). Smaller volumes of IgG were able to detect no less than 100 ng of flavocytochrome, and increasing the volume of IgG to greater than 100 µl did not result in an increased ability to detect flavocytochrome c. No binding of IgG occurred to the control protein BSA. This indicated that flavocytochrome specific IgG was specific enough to use for library screening subject to these predetermined conditions.

The library was screened as described in section 2.24.2. at a density of approximately 2000 colony forming units per plate. Development of the filters was controlled as described below. The control filter of MM294 containing non-recombinant pEX3 was developed first to determine the background development time (due to non-specific binding of the antibody). The experimental filters with recombinant colonies were then developed individually with any spot developing more quickly than the background recorded as corresponding to a putative positive clone (Plate 4.5.). Ten spots fitting this criterion were picked and found to correspond to colonies on alignment of filters with the original plates. This was a rather high number of putative positive clones as in a library of approximately 22 000 independent recombinants it would be expected to identify only one or perhaps two putative positive colonies.

The colonies giving positive hybridisation were isolated and rescreened according to section 2.24.2. On rescreening, only one of the original ten positive clones gave a strong hybridisation signal, which was more in agreement with the previous estimate of one or two positive clones.

This colony was further analysed as described in section 2.24.3. to investigate hybridisation of flavocytochrome c-specific IgG to any cellular protein. Western blotting showed several flavocytochrome c related protein

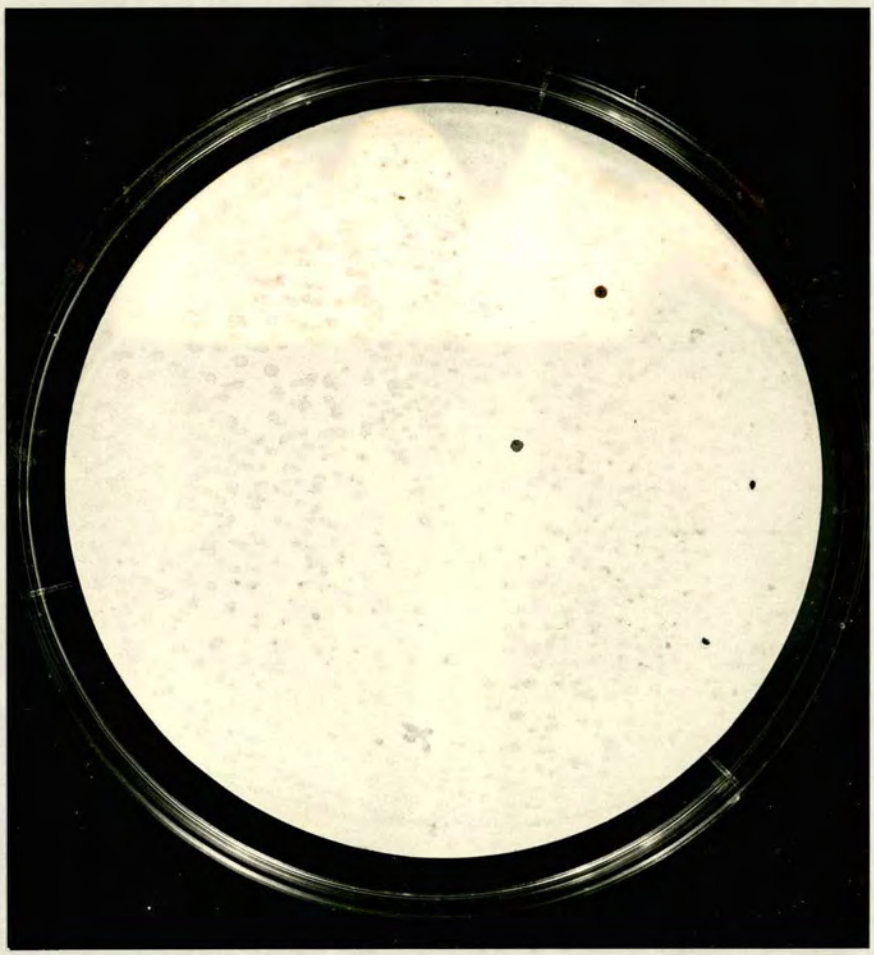
Plate 4.4. Specificity of flavocytochrome specific IgG.

1 2 3 4 5 6

Dilutions of purified flavocytochrome c were spotted on to nylon membrane. The membrane was air dried then developed according to section 2.12. with 100 µl IgG raised to purified flavocytochrome c.

Spot	amount of purified flavocytochrome (ng).
1	BSA (100 µg)
2	0.01
3	0.1
4	1
5	10
6	100

Plate 4.5. Colony screen of *S. putrefaciens* pEX3 expression library.



The pEX3 expression library in MM294 was plated at approximately 2000 colonies per 7.5 cm diameter plate. After incubation at 30^{o}C for 16 h followed by induction at 42^{o}C for 2 h, the colonies were replica plated onto hybond N and lysed by incubating at 95^{o}C for 30 min on a pad of filter paper presoaked in 5% SDS. Cell debris and SDS were removed by electroblotting (1 h at 50V) followed by washing in TBS containing lysozyme (10 mg ml^{-1}). The filters were then developed with flavocytochrome specific IgG.

bands expressed from the putative positive clone, the largest of which was approximately 45 kDa. (Plate 4.6.). Antibody did not recognise protein from other recombinants or non-recombinants cultured either at 30°C or 42°C, therefore excluding the possibility that antibody was recognising a heat shock protein induced during elevated incubation temperatures.

Recombinant plasmid was purified and the cloned fragment judged to be approximately 1.3-1.5 kb (Plate 4.9.), which should encode a protein of approximately 50-60 kDa. This size is similar to that of the largest protein band detected following development of the Western blot (Plate 4.6.). From the information on the size of the cloned fragment however, it could be presumed not to encode the full length flavocytochrome, as from the flavocytochrome molecular weight of 84 kDa, a DNA coding sequence of slightly over 2 kb would be required.

It was rather surprising that the antibody did not detect the induced recombinant protein as an hybrid protein (Stanley and Luzio, 1984). To determine whether this occurred only with this particular clone or if it was common to other clones in the library, a Western blot as in Plate 4.6. was performed, only this time was developed with anti β-galactosidase antibody. In all cases, hybridisation was observed to a 90 kDa protein which corresponded to truncated β-galactosidase from non-recombinant pEX3. No hybridisation was observed to a protein of more than 90 kDa and none to the proteins of 45 kDa and less, which bound flavocytochrome specific antibody. Furthermore Kenacid blue stained gels showed no protein bands corresponding to those produced by the recombinant plasmid which hybridised to flavocytochrome specific IgG.

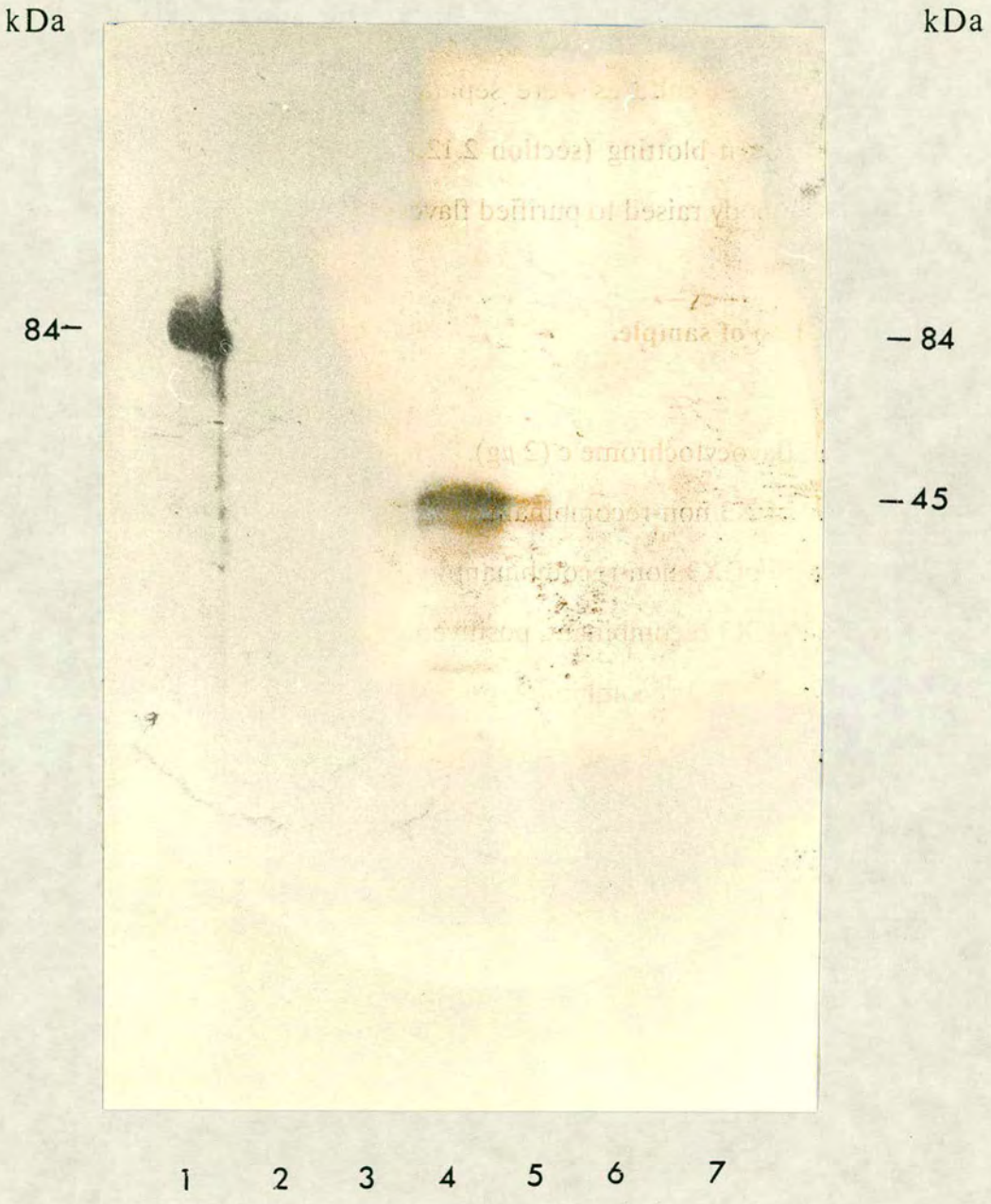
The literature (Stanley and Luzio, 1984) suggested that induced hybrid protein should have been readily observed as up to 25% of total cell protein following incubation at 42°C for 2 h. In this case, the low level of induced

Plate 4.6. Western blot of pEX3 recombinants.

Cultures were grown aerobically at 30°C in LB containing ampicillin and kanamycin to reach an OD_{600} of 0.4 then incubated at 42°C for 90 min to induce expression of hybrid protein. Total lysates (approximately 30 μg) of induced and uninduced cultures were separated by SDS-PAGE (10%), then transferred by Western blotting (section 2.12.) to nylon membrane which was developed with antibody raised to purified flavocytochrome c.

Lane	Description of sample.
1	purified flavocytochrome c (2 μg).
2	induced pEX3 non-recombinant.
3	uninduced pEX3 non-recombinant.
4	induced pEX3 recombinant, positive in both primary colony screens.
5	uninduced pEX3 recombinant, positive in both primary colony screens.
6	induced pEX3 recombinant, positive on the initial primary colony screen but negative on the second.
7	uninduced pEX3 recombinant, positive on the initial primary colony screen but negative on the second.

Plate 4.6. Western blot of pEX3 recombinants.



hybrid protein and its presence as several bands strongly suggested that it was subject to some slight proteolysis. This was further confirmed by the report that rapidly shifting a mid-exponential culture from 30°C to 42°C resulted in generalised heat shock, with some of the proteins induced being proteases (Baker *et al*, 1984). The failure of flavocytochrome c antibody to bind to protein corresponding to the truncated β-galactosidase indicated that the pEX3 hybrid was cleaved at the fusion junction following temperature induction, releasing two proteins, one corresponding to the *cro-lacZ* from pEX3 and the other protein originating from the cloned DNA fragment.

The induced cell lysate was haem stained to identify whether haem was incorporated into protein expressed from this cloned DNA, and stained by zymogram for fumarate reductase activity. No fumarate reductase activity was detected, and no haem staining bands corresponding to protein expressed from the cloned fragment were observed.

4.4. Characterisation of the cloned fragment

The flavocytochrome c gene fragment cloned in pEX3 was characterised by DNA sequence analysis in the expectation that comparison of the predicted amino acid sequence with other known sequences, particularly from flavoproteins and cytochromes, might allow identification of particular functional regions of the flavocytochrome c polypeptide. This information would be of great importance to subsequent biochemical and biophysical analysis of the structure and function of flavocytochrome c.

4.4.1. Genomic southerns of *S. putrefaciens* and *E. coli*.

To confirm that the fragment cloned in pEX3 originated from the genome of *S. putrefaciens*, hybridisation of the fragment to chromosomal DNA

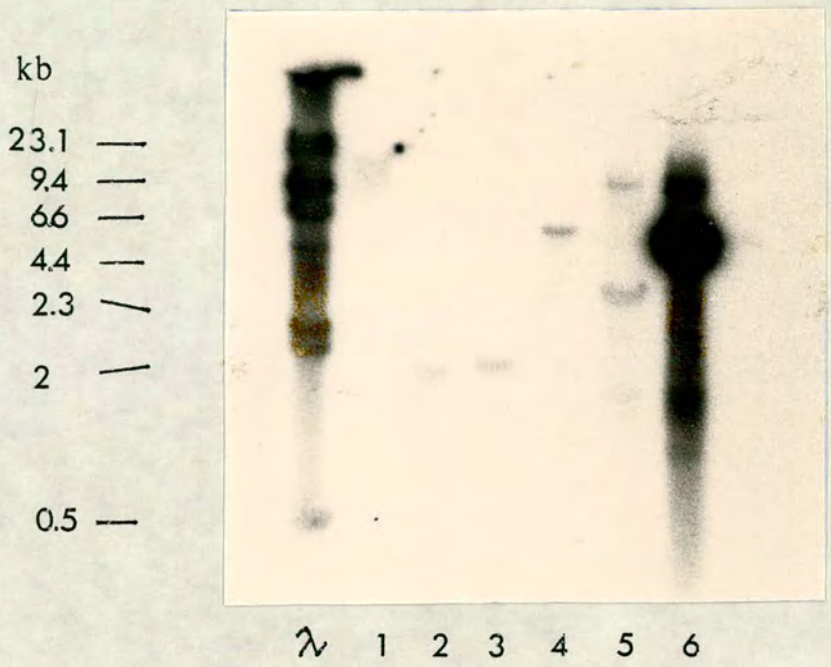
was investigated. Genomic DNA from *S. putrefaciens* and *E. coli* MM294 was digested independently to completion with several restriction enzymes, the fragments separated on agarose, then probed following Southern blotting. The fragment hybridised to a large (> 20 kb) *Bam*HI fragment, a 5 kb *Pst*I fragment, a 1.8 kb *Hind*III fragment and a 1.6 kb *Eco*RI fragment from *S. putrefaciens* (Plate 4.7.). No hybridisation was observed to any chromosomal fragment from *E. coli*. This indicated that the fragment was of *S. putrefaciens* genomic origin, and in addition provided preliminary mapping information of the fragment with respect to the flanking genomic regions.

4.4.2. Formation of sequencing constructs

To enable sequencing of the cloned flavocytochrome c gene fragment, the insert was then transferred from the recombinant pEX3 vector (pACB1) into phagemid vectors specifically designed for DNA sequencing. The vectors chosen for this were pTZ18R and pTZ19R (Rokeach *et al*, 1988) (Figure 2.3.) which are identical except that the polylinker is inserted in the inverse orientation in each vector, permitting sequencing of both DNA strands. The presence of both pBR322 and f1 origins of replication, permits the production of both double-stranded and single-stranded DNA (in the presence of helper phage M13K07).

To isolate the pEX3 fragment as a single band which could be cloned directly into the polylinker of pTZ18R and pTZ19R, pACB1 was digested with *Sma*I and *Pst*I which flanked the fragment in the polylinker and also cut uniquely in the polylinker of both sequencing vectors. These enzymes were considered not to have any sites centrally located in the fragment, as *SmaI/PstI* double digests of pACB1 yielded only two visible bands, one of 5.75 kb which was identical in size to the parent vector, and one of approximately 1.3-1.5 kb

Plate 4.7. Genomic Southern of *S. putrefaciens*.



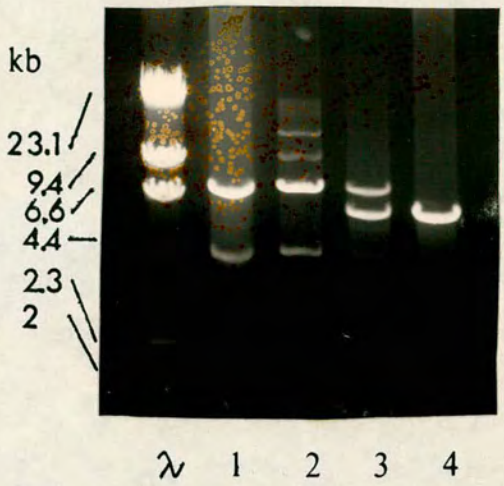
Chromosomal DNA (1 μg) from *S. putrefaciens* was digested to completion with restriction enzymes as listed below. Plasmid pTZ19R (500 ng) and pTZ19R containing the cloned fragment (500 ng) were digested with *Pst*I. The fragments were separated on agarose then probed with the cloned DNA fragment according to section 2.19..

Lane	DNA source	Enzyme
1	chromosome	*Bam*HI
2	chromosome	*Eco*RI
3	chromosome	*Hind*III
4	chromosome	*Pst*I
5	pTZ19R	*Pst*I
6	pTZ19R + cloned fragment	*Pst*I

which corresponded to the cloned fragment (Plate 4.8.). Furthermore, the reduction in pACB1 size (of approximately 1.5 kb) when doubly digested with *Pst*I and *Sma*I corresponded to the predicted insert size. Although the cloned fragment appears very faint in Plate 4.8., it can be observed more clearly in lane 7 of Plate 4.9. where it is more accurately sized by comparison to a *Bgl*I digest of pTZ19R which yields fragments of 1279 bp and 1571 bp. (The other bands of 2.1 kb and 2.8 kb are due to the plasmid pcI857 encoding the temperature sensitive repressor which controls expression of cloned DNA in pEX3).

The complete fragment was isolated by a *Sma*I/*Pst*I double digest of pACB1 then subcloned separately into the polylinkers of pTZ18R (Figure 4.2.) and pTZ19R (Figure 4.3.) to yield recombinants pACB2 and pACB3 respectively. Sequencing of the fragment in these vectors gave approximately 800 bp of sequence comprising of about 400 bp from either end with the centre stretch unsequenced. In an attempt to complete the sequence, the fragment was partially mapped in pACB3 with several enzymes to identify potentially useful internal restriction sites which would facilitate appropriate manipulation of the cloning constructs (see Figure 2.3. for sites of these enzymes within the parent vector). *Cla*I, *Dra*I, *Pvu*II and *Eco*RI were all found to cut within the fragment (Plate 4.9.). Of those, the only useful site seemed to be *EcoR*I which cut at a site approximately 500bp from the *Sma*I site. *Cla*I, *Pvu*II and *Dra*I were not as potentially useful, with *Cla*I having two restriction sites in the fragment, one at the *Sma*I end and one centrally located, and the *Dra*I and *Pvu*II sites being located 100 bp and 200 bp respectively again from the *Sma*I end of the fragment. An *Eco*RI deletion construct (pACB21) was made in pACB2 by removal of an internal 500 bp *Eco*RI fragment. The vector was then religated, thus transferring the internal *Eco*RI site to a position in the polylinker, which enabled sequencing from the internal *Eco*RI site (Figure 4.4.). Overlapping

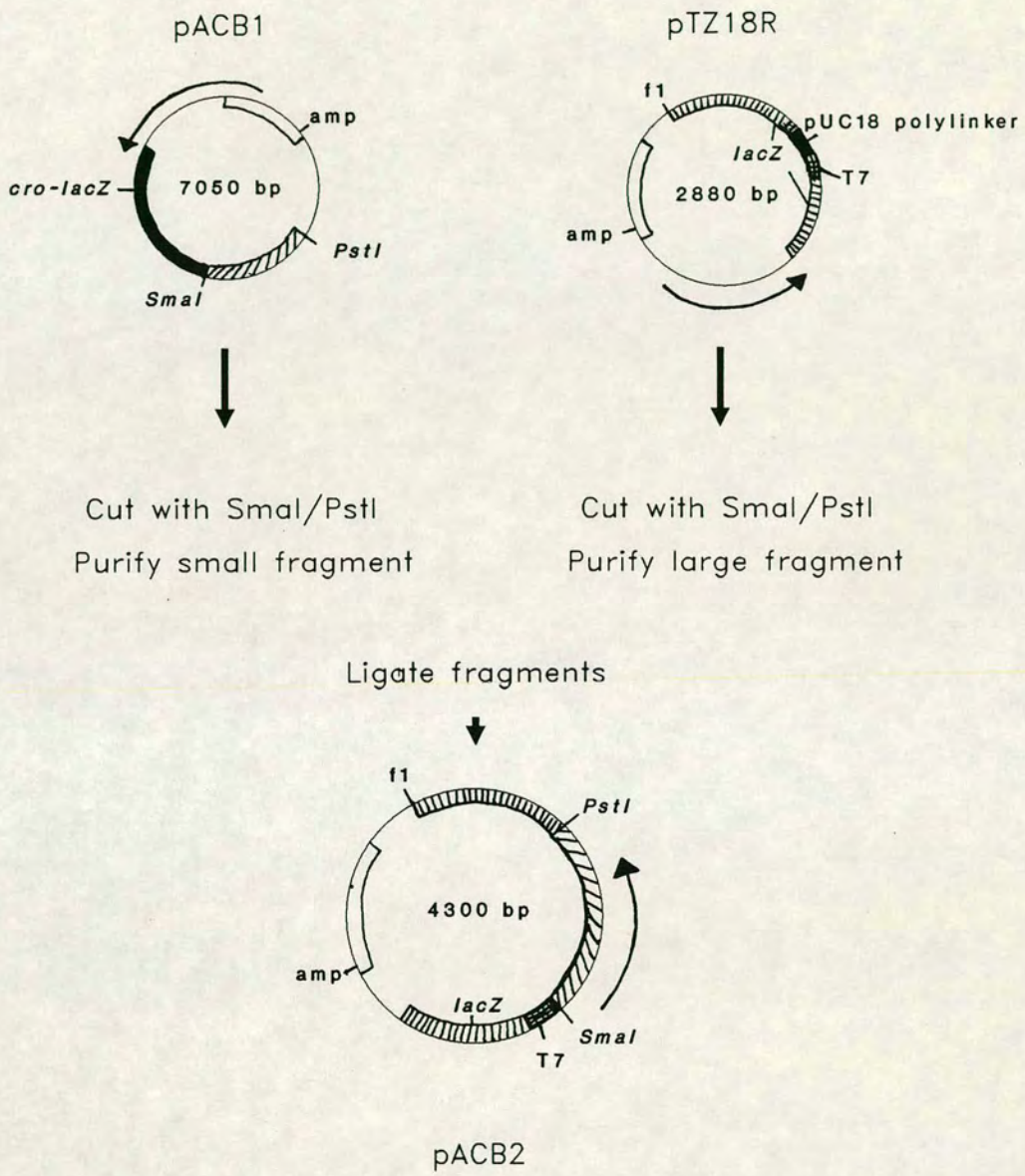
Plate 4.8. Isolation of the cloned fragment from pEX3.



Plasmid DNA (500ng) isolated from MM294 hosting either pEX3, or pEX3 recombinant containing the cloned fragment was digested as described below.

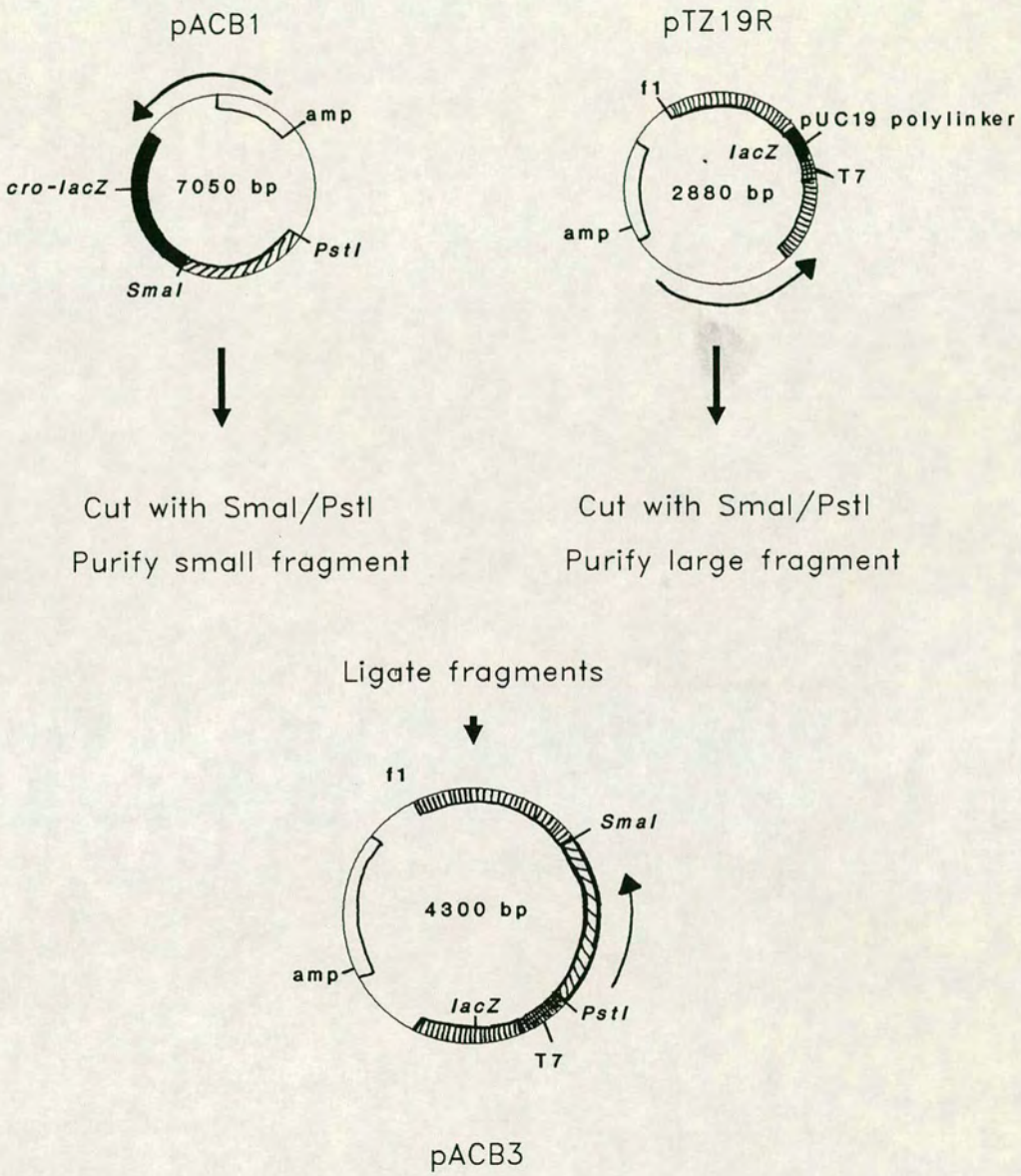
Lane	DNA source	Enzyme(s)
1	pEX3 recombinant	*Sma*I
2	pEX3 recombinant	*Pst*I
3	pEX3 recombinant	*Sma*I/*Pst*I
4	pEX3	*Sma*I/*Pst*I

Figure 4.2. Construction of pACB2.



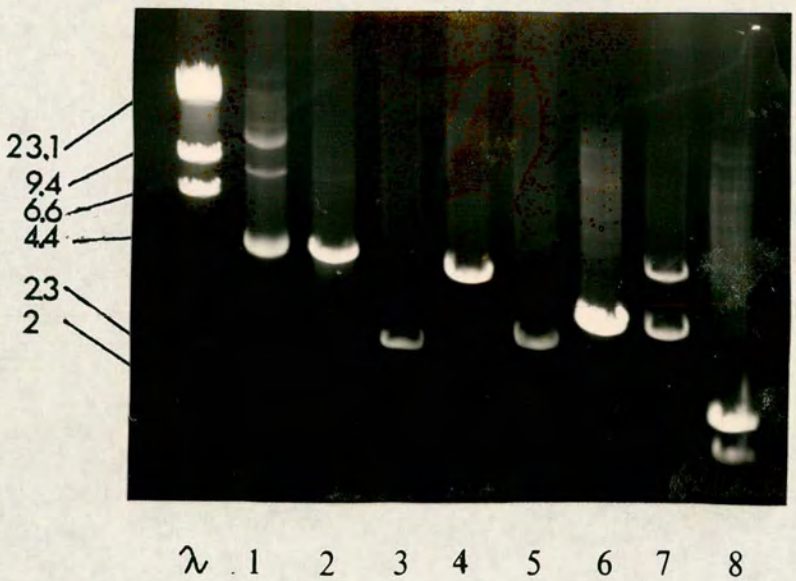
The small *SmaI/PstI* fragment from pACB1 (the pEX3 recombinant expressing protein which hybridised to IgG raised against purified flavocytochrome c) was ligated into pTZ18R digested with *SmaI/PstI* to create pACB2.

Figure 4.3. Construction of pACB3.



The small *SmaI/PstI* fragment from pACB1 (the pEX3 recombinant expressing protein which hybridised to IgG raised against purified flavocytochrome c) was ligated into ptZ19R digested with *SmaI/PstI* to create pACB3.

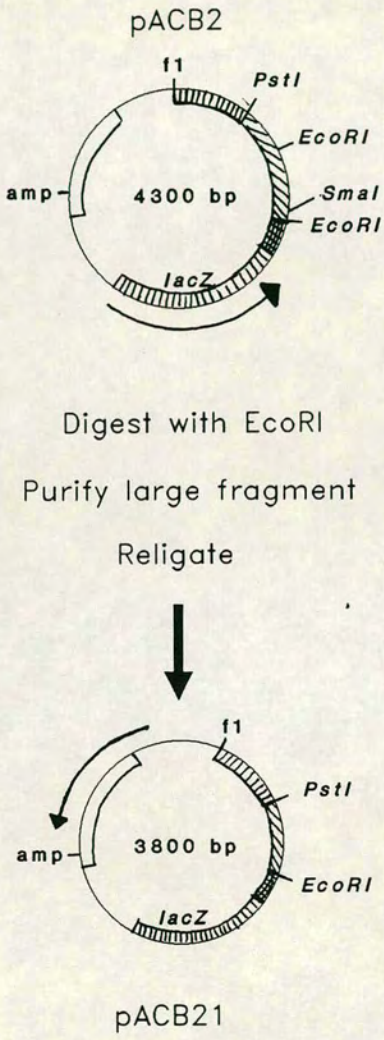
Plate 4.9. Mapping and sizing cloned fragment in pACB2.



Plasmid DNA (500 ng) was digested with the enzymes listed below prior to electrophoresis.

Lane	DNA source	Enzyme
1	pTZ19R	*Pst*I
2	pACB3	*Cla*I
3	pACB3	*Dra*I
4	pACB3	*Eco*RI
5	pACB3	*Pvu*II
6	pTZ19R	*Pst*I/*Sma*I
7	pACB3	*Pst*I/*Sma*I
8	pTZ19R	*Bgl*I

Figure 4.4. Construction of pACB21.



The 0.5 kb *EcoRI* fragment (approximate size) from pACB2 was deleted and the vector religated to generate pACB21.

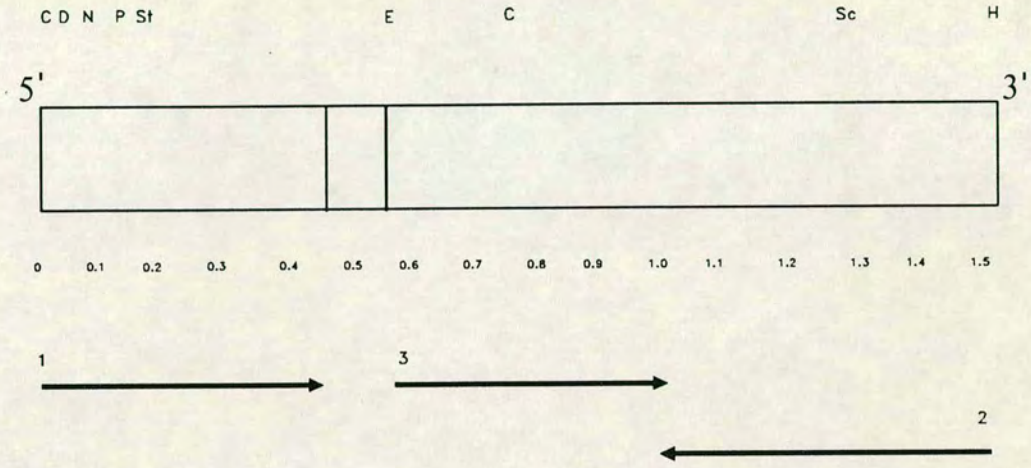
sequence was obtained from the constructs pACB3 and pACB21, but the sequence from pACB2 was not of sufficient length to give a complete overlap with that of pACB3. The sequencing strategy and partial restriction map is presented in Figure 4.5., with the nucleotide sequence of the two DNA fragments presented in Figure 4.6.

To identify the most likely translational reading frame, the sequences were translated in both directions and all reading frames using the computer program MAP of the UWGCG package of sequence analysis programs (section 2.18.3.). Frequent stop codons were obtained on translation of both parts of the sequence in five reading frames. The sixth reading frame (the same in both sequences) was tentatively suggested as the true reading frame as in addition to the lack of stop codons it corresponded in both orientation and frame to that from which the fragment was originally cloned in pEX3. The predicted amino acid sequence of the two fragments is presented in Figure 4.7.

4.4.3. Position of flavocytochrome c coding region within the cloned fragment.

At this stage it was not known to which region of the flavocytochrome gene the fragment corresponded nor how much upstream or downstream sequence was present in addition to the coding region. In order to position the flavocytochrome coding region within the fragment, it was decided to compare the amino acid sequence from purified protein with the translated DNA sequence. Correspondence of the N-terminal amino acid sequence with a region of the fragment would establish the location of the start of the flavocytochrome coding region. In the absence of any identity with the N-terminal sequence, identification of the coding domain could be achieved by comparison of internal protein sequence (by sequencing of peptide fragments

Figure 4.5. Partial Restriction map and Sequencing Strategy of Cloned Fragment.



1 = Sequence from ACB2.
2 = Sequence from ACB3.
3 = Sequence from ACB21.

C = *ClaI*
D = *DraI*
N = *NruI*
P = *PvuII*
St = *StuI*
E = *EcoRI*
H = *HindIII*
Sc = *ScaI*

Figure 4.6. Nucleotide sequence of the cloned fragment.

(i)

```
1      atgcgcac acaagttccg cttaatcgat tgaaaaaaca tctttttttt aaagatgtgt tcgatgctgt gtacctgtgc
81   tcacagaggt ctacttttttc gcgaaaacgt agatctctac cgcccaacga aaagcatgaa agcgatcacg aatcccattg
161  ggtcgtcatg ttctgccgat cgcatcttcc tatcctcgct ccaggcctgc cgcataacca atcaggcttc ctacttacag
241  aattgagaaa agaggatgtg gaaatgctca gtcaaattca accgctttgg ctgcgcgatg ttcacgccag gtctgctgtt
321  tcccttcgcc gggattgtgg tgggtcttgc catcttgctg caaaacccga tgtttgtcgg gaatcactga ccgatccgaa
401  cagtttattc gcgcaaatcg tacacattat tgaagaggcg t
```

(ii)

```
1    gaattccatg tacaaaacca agaatgtgat agctgccata caccagatgg tgaactgtca aacgacagct taacctatga
81   aaatacccaa tgcgtatctt gccatggcac acttgctgaa gtagctgaaa ccacaaaaca tgaacattat aatgctcatg
161  cttctcattt ccctggcgaa gtagcttgta cctcatgcca cagcgcacac gaaaaatcga tggtgtattg tgactcttgc
241  cacagcttcg atttcaacat gccttatgct aaaaaatggc tacgtgacga gccgactatt gctgaattgg ccaaagacaa
321  atcagaacgt caggctgctc ttgctagcgc acctcacgat actgttgacg tagtggttgt cggttctggc ggcgcaggtt
401  tctcagcggc aatatcagca acagacagtg gtgctaaagt cattcttatt gaaaaagagc ctgttattgg tggtaatgct
481  aagttagctg cgggtggcat gaacgctgct tggactgatc aacaaaagcc aaaaaaaatt actgacagcc cagagttaat
561  gttcgaagac accatgaaag gtggccaaaa cataaatgac cctgcattag ttaaagtatt aagctcacac tctaaagact
641  ctgttgattg gatgaccgct atgggtgccg atttaactga tgttggcatg atgggtggcg catctgttaa tcgtgcgcat
721  cgtccaaccg gtggtgcggg tgttggtgct catgttgttc aagtacttta tgataatgca gtgaaacgca atatcgactt
801  acgcatgaac actcgcggca ttgaagtgct taaagatgat aaaggcactg ttaaaggtat tctggttaag gggatgtaca
881  aaggttacta ctgggtgaaa gccgatgcgg taatcttagc aacgggtggt ttccgctaaa aataacgagc gtgtcgccaa
961  agcttgat
```

(i) Upstream sequence from the 5' end of the fragment in pTZ19R.

(ii) Overlapping coding sequence obtained from the 5' *EcoRI* internal site in pTZ19R and the 3'end in ptZ18R.

Figure 4.7. Predicted amino acid sequence of the cloned fragment and N-terminal sequence of purified flavocytochrome c.

(i)

1 MRTQVPLNRL KKHLFFKDVF DAVYLCSQRS TFSRKRRSLP PNEKHESDHE

51 SHWVVMFCRS HLPILAPGLP HNQSGFLLTE LRKEDVEMLS QIQPLWLRDV

101 HARSAVSLRR DCGGSCHLAA KPDVCRESLT DPNSLFAQIV HIIEEA..

(ii)

1 EFHVQNQECD SCHTPDGELS NDSLTYENTQ CVSCHGTLAE VAETTKHEHY

51 NAHASHFPGE VACTSCHSAH EKSMVYCDSC HSFDFNMPYA KKWLRDEPTI

101 AELAKDKSER QAALASAPHD TVDVVVVGSG GAGFSAAISA TDSGAKVILI

151 EKEPVIGGNA KLAAGGMNAA WTDQQKPKKI TDSPELMFED TMKGGQNIND

201 PALVKVLSSH SKDSVDWMTA MGADLTDVGM MGGASVNRAH RPTGGAGVGA

251 HVVQVLYDNA VKRNIDLRMN TRGIEVLKDD KGTVKGILVK GMYKGYYWVK

301 ADAVILATGG FAKNNERVAK LD...

(iii)

1 AQNLAEFHVQNQE÷D

(i) Predicted amino acid sequence upstream of the flavocytochrome c coding region.

(ii) Predicted amino acid sequence of flavocytochrome c coding region downstream of the *EcoRI* site. The four predicted heme attachment sites are underlined and the consensus sequence for FAD binding sites is double underlined.

(iii) Amino acid sequence obtained on N-terminal sequencing of flavocytochrome c. The underlined region corresponds to the sequence predicted from the DNA sequence from the internal *Eco*RI site as in (ii) above.

generated by specific proteolytic cleavage of purified flavocytochrome) with the translated DNA sequence.

N-terminal sequencing of flavocytochrome c yielded thirteen residues (Figure 4.7.(iii)) which on comparison with the translated fragment sequence gave complete identity from residues 6-13 of the protein sequence to a region starting at and continuing downstream from the *Eco*RI site (Figure 4.7.). This suggested the start of the flavocytochrome coding region to be located within the cloned fragment. Therefore approximately two thirds of the fragment, from a region just preceding the *Eco*RI site downstream to the fragment end comprised of flavocytochrome coding region with the remainder constituting upstream sequence.

4.5. Molecular analysis of the cloned fragment.

The flavocytochrome c amino acid sequence was compared to all other protein sequences in the Swissprot and OWL Databases. Flavocytochrome c showed similarity to both c-type cytochromes and flavoproteins, indicating the presence of two distinct domains.

4.5.1. Haem domain

On the basis of a low redox potential and multiple haems, the flavocytochrome was previously suggested by Morris, (1987) to be a ClassIII type cytochrome-c (Pettigrew and Moore, 1987).

In this study, the predicted amino acid sequence identified four haem attachment sites within 90 residues of the N-terminus, all of the motif C-X-Y-C-H which is a well conserved sequence among c-type cytochromes. This region was identified as the flavocytochrome c haem domain. All the Y residues were conserved as serine with X residues less conserved, being aspartate in two cases,

valine and threonine in the others. Identity of the flavocytochrome c haem binding domain was observed with other c-type cytochromes but was limited to the haem attachment sites.

The flavocytochrome c haem domain was compared with sequences from other ClassIII type cytochromes such as the mutihaem cytochromes c_3 from *D. desulfuricans*, *D. gigas* and *D. vulgaris* (Ambler *et al*, 1971) and the c_7 cytochrome from *Chl. ethylica* (Ambler, 1971).

The c_3 cytochromes were each of similar size (12 kDa) and contained four haem attachment sites within 100 residues, two with the characteristic motif C-X-Y-C-H, and two of the arrangement C-X-Y-X-Y-C-H. Alignment of the c_3 amino acid sequences showed that the haem binding sites occurred at almost identical regions of the sequence in each cytochrome with sequence homology highly conserved in the early part of the sequence. Cytochrome c_7 contained three haem binding sites within a 68 amino acid peptide sequence which showed some similarity to that of *Desulfovibrio*, especially to that of *D. desulfuricans*, in regions between the haem binding sites. In agreement with the c_3 and c_7 cytochromes, the flavocytochrome haem binding domains occurred within the first 100 residues near the protein N-terminus. There were no regions of identity between these cytochromes and the flavocytochrome apart from the characteristic haem binding site motif, and no alignment similarity of the haem domains.

4.5.2. Flavin domain

There was a high region of sequence conservation from residue 123-151 of flavocytochrome c with the N-terminal region of other flavoproteins (Figures 4.7ii, 4.8. and 4.9.). This region encompassing approximately 32 residues was previously described as the contact site for the bottom of the adenine portion of

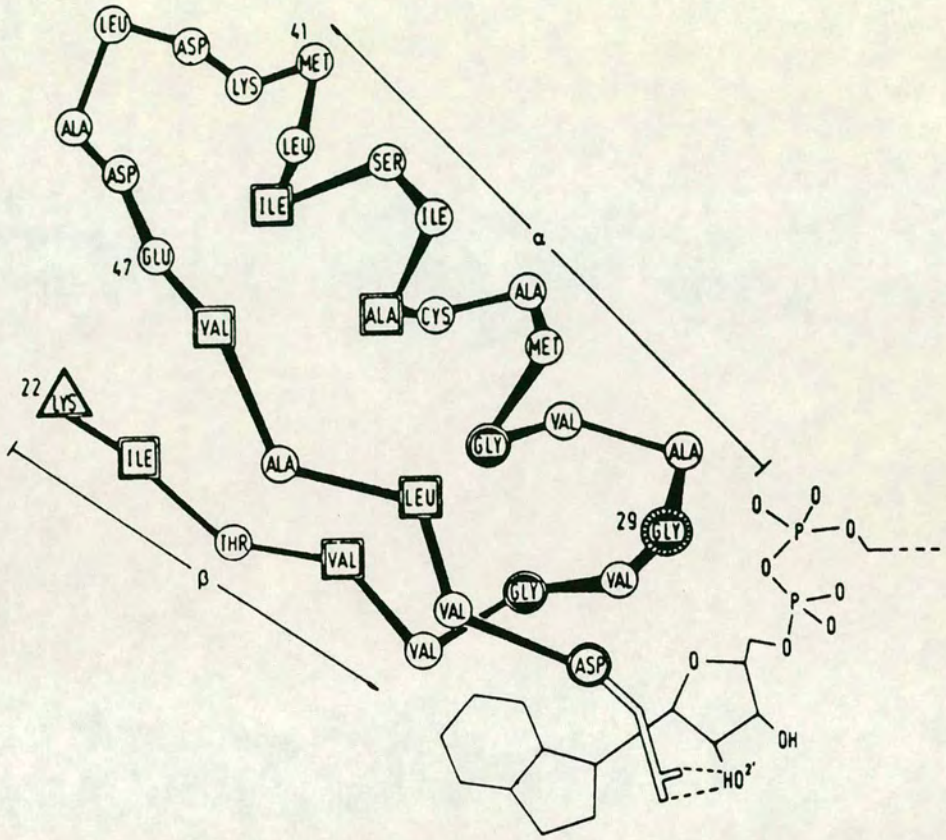
the FAD in FAD-binding folds (Williams *et al*, 1978). An amino acid fingerprint which allowed folding of a general flavoprotein, in a $\beta\alpha\beta$ fold, in such a way to enable it to bind adenine was later reported by Wierenga *et al* (1986). It has been suggested that the $\beta\alpha\beta$ stucture, which always occurs near the N-terminus of the dinucleotide binding domains, may function in the tertiary structure of the protein as a nucleation centre for the folding of the complete domain (Schulz and Schirmer, 1979).

The predicted general structure (Figure 4.8.) was a $\beta\alpha\beta$ unit of 29-31 residues with 11 conserved residues, each occurring at a specific position, allowing the peptide to form a $\beta\alpha\beta$ configuration (the variable length of the peptide accounted for by a loop between the α helix and the second β strand which could vary in residue number from 2-4). The conserved residues were arranged as a characteristic stretch of 4 hydrophobic residues followed by a glycine residue (β_A), a stretch of short side chain residues (α_A) and another region of hydrophobic residues (β_B). The important residues were thought to be the 3 glycines, essential for folding of the protein into α helices.

The predicted general sequence was compared to the proposed flavocytochrome c FAD binding site in Figure 4.9. Similarity of flavocytochrome c to the fingerprint was obtained for all eleven conserved residues. This strongly confirmed at the molecular level that the flavocytochrome c flavin prosthetic group was FAD.

As flavocytochrome c was shown to have both fumarate reductase and succinate dehydrogenase activities (Chapter 3), the flavocytochrome c sequence was compared in detail with the amino acid sequences of the flavoproteins fumarate reductase (Cole, 1982) and succinate dehydrogenase from *E. coli* (Wood *et al*, 1984) and the succinate dehydrogenase from *B. subtilis* (Phillips *et al*, 1987) all of which showed approximately 30% identity to flavocytochrome c

Figure 4.8. General ADP-binding βαβ-fold.



The spiny dogfish M-lactate dehydrogenase (Taylor, 1977; Eventoff *et al.*, 1977) was used to describe the general structure of the ADP-binding βαβ-fold (Wierenga et al., 1986).

The highly conserved fingerprint residues are framed by two lines (△, basic or hydrophilic, □, small and hydrophobic, ○, glycine).

Figure 4.9. Comparison of flavocytochrome c FAD adenine binding region with other flavoproteins.

1	2	3	4	5	6	7	8	9	10	11
*	*	*	*	*	*	*	*	*	*	*
D	V	V	V	V	G	S	G	G A G F S A	A	I S A T D S G A K	V	I	L	I	E

Similarity of the predicted flavocytochrome c adenine binding region with an amino acid fingerprint for FAD and NAD binding sites.

Highly conserved residues are marked with an asterisk with corresponding residues of flavocytochrome c underlined.

Key to conserved positions.

Residue	type of base
1	basic or hydrophilic
2,3,7, 8,9,10	small and hydrophobic
4,5,6	glycine
11	acid

Figure 4.10 Alignment of flavocytochrome c flavin domain with flavin subunits from E. coli fumarate reductase and succinate dehydrogenase, and B. subtilis succinate dehydrogenase

```
                          x      x xxx* *  x**  xxx x    x   xxx   *x x xx      46
Flavocytochrome c  ····ASAPHD  TVDVVVVGSG  GAGFSAAISA  TDS·G·AKVI  LIEKEPVIGG
E. coli FrdA             QTF  QADLAIVGAG  GAGLRAAIAA  AQANPNAKIA  LISKVYPMRS
E. coli SDH           MKLPVR  EFDAVVIGAG  GAGIARALQI  SQSGQ··TCA  LLSKVFPTRS
B. subtilis SDH            S  QSSIIVVGGG  LAGLMATIKA  AESG··MAVK  LFSIVPVKRS

                     * ** x  x*     x      *xxx  x  ** x*x  x   x   xx      96
                   NAKLAAGGMN  AAWTDQQKPK  KITDSPELMF  EDTMKGGQNI  NDPALVKVLS
                   HTVAAEGG·S  AAVAQ···DH  ···DSFEYHF  HDTVAGGDWL  CEQDVVDYFV
                   HTVSAQGGIT  VALGN···TH  ··EDNWENHM  YDTVKGSDYI  GDQDAIEMYC
                   HSVCAQGGIN  GAVN··TKGE  ··GDSPWEHF  DDTVYGGDFL  ANQPPLKAMC

                    x          x                        xx   **x  x       x  146
                   SHSKDSVDWM  E·········  ····TAMQAD  LTDVGMMGGA  S·····VNRA
                   HHCPTEMTQL  ELWGCPWSRR  PDGS·····  ·VNVRRFGGM  ·······KIE
                   KTGPEAILEL  EHMGLPFSRL  DDGR·····  ·IYQRPFGGA  SKNFGGEQAA
                   EAAPSIIHLL  DRMAVMFNRT  PEG·····  LLDFRRFGGT  ·······QHH

                       xx *   x    *x     x          x          xxxx x  196
                   HRPTGGAGVG  AHVVQVLYDN  AVK·····  RNIDLRMNTR  GIEVLKDDKG
                   RTWFAADKIG  FHMLHTLFQT  SLQF···PQI  QRFDEHF···  VLDILV·DDG
                   RTAAAADRTG  HALLHTLYQQ  NLK····NHT  TIFSEWY···  ALDLVKNQDG
                   RTAYAGATGG  QQLLYALDEQ  VRRYEVAGLV  TKYEGWE···  FLGAVLDDDR

                   xx *x    x    x     xx  x*xxx****                        237
                   TVKGILVKGM  YKGYYW·VKA  DAVILATGGF  AKNNERVAKL  D·
                   HVRGLVAMNM  MEGTLVQIRA  NAVVMATGGA  GR·····  ·
                   AVVGCTALCI  ETGEVVYFKA  RATVLATGGA  GR·····  ·
                   TCRGIVAQNL  TNMQIESFRS  DAVIMATGGP  GI·····  ·
```

*: Conservation of residue in all four proteins

x: Conservation of residue in flavocytochrome c and one or two of the other flavoproteins

over a more extensive region of approximately 200 amino acid residues directly downstream of the FAD binding region. Covalent binding of FAD depends on the presence of a highly conserved histidine residue occurring 42 residues downstream of the N-terminus. This residue was not conserved in flavocytochrome c further confirming earlier experimental observations (Morris, 1987) that the flavin was non-covalently bound.

The active site cysteine at residue 87 in the flavoproteins was not conserved in flavocytochrome c. Close examination of the available flavocytochrome sequence failed to identify any potential active site cysteine residues in the proposed flavin domain, which was considered rather unusual as previous studies of flavoprotein subunits have indicated that thiol groups are essential for activity, substrate binding, or both (Kenney *et al*, 1976; Robinson and Weiner, 1982; Unden and Kroger, 1980).

Identification of other active site cysteines and histidines, in particular a His-Pro-Thr sequence which may be conserved in the active site of other flavoproteins (Rice *et al*, 1984), could not be done as the flavocytochrome c sequence had not been extended this far. Very few histidines were observed in the available sequence from flavocytochrome c, none of which corresponded to conserved residues in the other flavoproteins. The importance of histidine has not yet been fully identified although they are considered to perform proton donor-acceptor functions in succinate dehydrogenase (Hederstedt and Hatefi, 1986, Vik and Hatefi, 1981). There were no other readily identifiable regions of active site similarity between flavocytochrome c and these other enzymes, so the observed identity of 30% over 200 residues was thought to be structural and involved in protein folding to allow substrate binding.

Although the complete flavocytochrome c coding sequence is not yet available, by examining the conserved regions of other flavoproteins it was

considered possible to predict some function for the remaining unsequenced region. It was indicated from the molecular weight of other flavoproteins (the flavoprotein subunit of *E. coli* fumarate reductase is 68 kDa; that of the *B. subtilis* succinate dehydrogenase is 65 kDa) that the remainder of flavocytochrome c (of approximately 74 kDa; 10 kDa being the haem domain) would be probably be the flavin domain. In particular, the unsequenced region downstream of the flavin domain would be expected to contain sequence involved in binding the top part of the FAD adenine. A region corresponding to this and located in residues 355-374 of *B. subtilis* succinate dehydrogenase is conserved in the flavoprotein subunits of both *E. coli* fumarate reductase (Cole et al, 1985) and succinate dehydrogenase (Wood *et al*, 1984), glutathione reductase and lipoamide dehydrogenase (Rice *et al*, 1984). This region is thought to bring the adjacent segment residues close to the flavin and active site.

4.5.3. Upstream region.

The nucleotide sequence (i) of Figure 4.6., which seemed to be open reading frame, was examined in an attempt to identify a promoter sequence. No consensus -35 (TTGACA) or -10 (TATAAT) regions were identified, nor were other less common promoter sequences (Lewin, 1990). It would seem likely therefore that the promoter was present in the 200 bp unsequenced region (Figure 4.5.) upstream of the predicted protein N-terminus, haem and flavin domains.

4.6. Screening for a clone encoding the entire flavocytochrome c gene

In parallel with sequencing, the cloned fragment was used as a homologous DNA probe to simultaneously screen the pUN121 (Chapter 5) and

pEX3 libraries in an attempt to isolate at least one further clone which encoded the full length flavocytochrome.

This was unsuccessful as although three thousand independent recombinants from each library were screened (according to section 2.19.1.2. and 2.19.3.) no colony was isolated encoding the full length flavocytochrome c.

CHAPTER 5

ATTEMPTS TO CLONE FLAVOCYTOCHROME C BY GENETIC COMPLEMENTATION

5.1. Preliminary Studies.

Preliminary studies were executed to establish which systems of genetic transfer could be easily developed to allow the introduction of DNA fragments into *S. putrefaciens*. The availability of such a system would enable the direct cloning of flavocytochrome c by functional complementation of a fumarate reductase mutation, the major advantage of this being isolation of a complete gene. Earlier work by Gibson (1981) showed conjugative transfer of the broad host range plasmid RP4 from *E. coli* to *S. putrefaciens* to occur at a frequency of 2×10^{-2} per recipient. Later studies (Campbell, 1986) involved the isolation and partial mapping of a 6.75 kb cryptic plasmid fom *S. putrefaciens*. This work showed that genetic transfer between *E. coli* and *S. putrefaciens* was possible and that genetic transfer also occurred within *S. putrefaciens* itself.

The early sections of this chapter investigate the possibility of cloning flavocytochrome c in *S. putrefaciens* itself by identification of a suitable cloning vector and method of gene transfer, in parallel with generation of a flavocytochrome c mutant and development of a screen for functional complementation of fumarate reductase activity. Later studies describe the attempt to clone the entire flavocytochrome c gene in *E. coli* by functional complementation of a characerised fumarate reductase mutant.

5.1.1. Transformation of *S.putrefaciens*

At the time of this study, no vectors designed specifically for use in *S. putrefaciens* were available to investigate transformation. In contrast, many cloning vectors have been constructed for *E. coli*, so it was decided to study the ability of *S. putrefaciens* to be transformed by and maintain the *E. coli* plasmid vector pAT153 (Twigg and Sherratt, 1980). pAT153 is a pBR322 derived 3.7 kb plasmid, which confers ampicillin and tetracycline resistance on the host.

Prior to attempting transformation, the inherent resistance of *S. putrefaciens* to various antibiotics was investigated to determine a suitable antibiotic for selection of transformants (Table 5.1.). *S. putrefaciens* NCMB400 was found to be sensitive to low levels of all antibiotics tested including ampicillin and tetracycline, which are encoded by pAT153. Therefore either ampicillin or tetracycline would be suitable for plasmid selection following transformation.

Transformation of *S. putrefaciens* was performed as follows. Mid exponential cultures were made putatively competent by a slight modification of the method described in section 2.18., which involved heat shock of the cells at 30°C and 25°C to account for the lower growth temperature of *S. putrefaciens*. Consultation of various articles showed heat shock of Gram-negative bacteria was generally carried out on mid to late exponential cultures at temperatures approximately 5°C above the optimal growth temperature (Spizizen, *et al*, 1966; Cohen *et al*, 1972). Plasmid pAT153 was then mixed with putatively competent cells and transformants selected from the cells shocked at each temperature, following non-selective expression at 2-hour intervals from 1 hour to 24 hours at 20°C, on nutrient agar plus 1% NaCl containing twice the *S. putrefaciens* growth inhibiting concentration of either tetracyline or ampicillin. Non-selective expression was allowed for this length of time as it was considered that due to the slower growth rate of *S. putrefaciens* an extended incubation might be required for expression of plasmid DNA, and resistance to the selective antibiotics was assessed separately in case one plasmid encoded resistance was not expressed as efficiently as the other preventing growth of transformants.

Transformation of *E. coli* HB101 by pAT153 was performed in parallel with that of *S. putrefaciens* using the standard procedure (section 2.18.) and transformants selected in duplicate on L-agar incorporating either ampicillin or

Table 5.1. Indigenous antibiotic resistance of *S. putrefaciens.*

Antibiotic	MIC ($\mu g\ ml^{-1}$)
tetracycline	5
ampicillin	5
kanamycin	10

Antibiotics (tetracycline, ampicillin, or kanamycin) were incorporated into nutrient agar, containing 1% NaCl, at concentrations ranging from 1 μg to 50 μg. The plates were incubated for 3 days at 20^{o}C following which the MIC (minimum inhibitory concentration) was defined as the concentration of antibiotic above which growth of all colonies was inhibited.

tetracycline at concentrations according to Table 2.3. Viable counts of both organisms were evaluated on non-selective media following transformation; in addition, the viability of *S. putrefaciens* was studied following treatment with calcium chloride and heat shock to determine any significantly damaging effect on the cells by this procedure. The cells remained viable throughout and the results of the transformation are presented in Table 5.2. No transformants were obtained from *S. putrefaciens* by selection on ampicillin or tetracycline following heat shock at either temperature. The possible reasons for unsuccessful transformation are discussed below (Section 5.1.4.).

5.1.2. Indigenous plasmid analysis in *S. putrefaciens*

Two plasmids of approximately 6.75 kb and 60 kb were previously isolated from *S. putrefaciens* D44, so plasmid profiles of *S. putrefaciens* NCMB400 were obtained to investigate the presence of indigenous plasmid which could prospectively be used as a cloning vector. No small indigenous plasmid was isolated from NCMB400 although there seemed to be a large (>20 kb) plasmid, of similar size to that observed in D44.

5.1.3. Generation of mutants in *S. putrefaciens*

Until recently the only methods available for mutagenesis in *S. putrefaciens* involved the use of chemical mutagens such as nitrosoguanidine which was used by Nasser (1983) to isolate electron transport mutants of *S. putrefaciens*. Although nitrosoguanidine is a potent mutagen, it preferentially mutates DNA which is replicating (Mandelstam et al, 1982) and often results in a series of closely linked mutations rather than a single mutation. Conventional techniques of transposon mutagenesis were restricted either due to the difficulty of introducing the transposon into *S. putrefaciens* or stability of mutations. The

Table 5.2. Transformation of *S. putrefaciens* NCMB400 and *E. coli* HB101 with pAT153.

	transformants	
Selection	**HB101**	**NCMB400**
ampicillin	3×10^5	0
tetracycline	2.9×10^5	0

pAT153 (30 ng) was mixed with putatively competent cells of *S. putrefaciens* (section 2.20.1.). Following heat shock (in duplicate at 25°C and 30°C) non-selective expression of DNA was allowed for up to 24 h at 20°C. Transformants were detected by selection for plasmid encoded antibiotic resistance on nutrient agar plates, containing 1% NaCl, plus either ampicillin or tetracycline at twice the MIC concentration (10 μg ml^{-1}). *E. coli* HB101 was transformed in parallel with 30 ng pAT153 by the standard transformation procedure (section 2.20.2.).

recent availability of the transposable element Tn5 on conjugative plasmids such as Omegon Km (Felley *et al*, 1989), has facilitated the stable mutation of single, random sites on the genome and as such are very useful for generating mutants in bacteria like *S. putrefaciens* which have no simple transformation system. Approximately 2000 mutants of *S. putrefaciens* generated by Omegon Km transposon mutagenesis have been screened for inactivation of the flavocytochrome c gene by the inability to utilise fumarate as terminal electron acceptor (Perry, 1989; Green, 1990). This was assessed by the development of an agar plate overlay assay where mutants were grown anaerobically then transferred to an anaerobic jar to simulate oxygen limited growth conditions. Following incubation for several hours, the plates were overlaid with agar containing fumarate and artificially reduced benzyl viologen. Colonies which could not utilise fumarate as a terminal electron acceptor would be unable to oxidise the reduced benzyl viologen thus remaining coloured. No fumarate reductase mutants have yet been isolated. A flavocytochrome c mutant would be expected to be defective in both TMAO and fumarate reduction, and to lack the 84 kDa flavocytochrome c protein following SDS PAGE. Complementation of such a mutation would result in functional restoration of both fumarate and TMAO reductase activity.

5.1.4. Conclusions

Transformation of *S. putrefaciens* was unsuccessful with the vector pAT153 (section 5.1.1.). This failure seemed not to be due to the damaging effect of some experimental stage to the cells, as viablity was maintained throughout the transformation procedure, but to either impermeability of the cells to the plasmid or instability of the plasmid within the cell resulting in its failure to replicate and eventual loss. The pAT153 origin of replication has a

narrow host range which prevents it from being maintained in a range of bacteria, although it was considered that as *S. putrefaciens* belongs to the same 5S ribosomal RNA family as *E. coli* and is therefore not too distantly related, pAT153 might be maintained in *S. putrefaciens*. Expression of the selective antibiotics ampicillin and tetracycline was not considered to be a problem as *S. putrefaciens* is able to express plasmid encoded antibiotic resistance (Gibson, 1981). It was reported that on conjugative transfer of RP4 from *E. coli* to *S. putrefaciens*, plasmid encoded resistance to tetracycline and ampicillin was expressed to a level well above the selective concentration in this experiment.

Although it seemed likely that the lack of transformants was due to the inability of pAT153 to be maintained in *S. putrefaciens*, it cannot be disregarded that calcium chloride treatment may have been ineffective at inducing competence in *S. putrefaciens*. Techniques used to facilitate the uptake of plasmid DNA by bacterial cells resistant to calcium chloride treatment have been described. They include sphaeroplasting and electroporation and are described below in more detail.

The transfer of DNA into sphaeroplasts generated by cell wall digestion by lysozyme was limited due to the difficulty of cell regeneratation. However a method involving the isolation of temperature sensitive peptidoglycan mutants by nitrosoguanidine was developed (Suzuki and Szalay, 1980). This involved the introduction of plasmid DNA into sphaeroplasts at the restrictive temperature of 42^{o}C (when no peptidoglycan synthesis was possible) followed by regeneration of cell walls at the permissive temperature of 30^{o}C. Although developed initially in *E. coli*, this procedure could be adapted for any bacterial species whose cell wall contained peptidoglycan.

Electroporation, involving the rapid polarisation of the cell membrane by a high voltage, results in a physical breakdown of the membrane (Zimmerman,

1982), which is associated with a reversible increase in membrane conductivity and permeability. It has been used to introduce DNA into a range of bacteria including *Lactobacilli* (Chassy and Flickinger, 1987), *Campylobacter jejuni* (Miller *et al*, 1988), and *Streptococci*, (Harlander, 1987; Powell *et al*, 1988), yielding up to 10^{10} transformants per ug DNA.

The ability of *S. putrefaciens* to take up plasmid DNA by these methods could be assessed with a broad host range vector such as the Inc W plasmid pGV1106 (Leemans *et al*, 1982) which was designed for cloning DNA fragments into transformable Gram-negative bacteria and confers resistance to kanamycin and streptomycin.

The construction of a cloning vector using an indigenous plasmid from *S. putrefaciens* was considered. Although no plasmid of suitable size was isolated from *S. putrefaciens* NCMB400, a plasmid was previously isolated from *S. putrefaciens* D44 of approximately 6.75 kb (section 5.1.2.). To allow the use of this plasmid as a vector, extensive characterisation by mapping and genetic manipulation would be required to insert a selective characteristic such as antibiotic resistance, to introduce single restriction sites suitable for use in cloning, and to ensure that throughout the manipulation the plasmid origin of replication was retained.

Plasmids such as pRK290, a derivative of RP4, (Ditta *et al*, 1980) have been constructed specifically to assist gene cloning in non-transformable bacteria. This plasmid can be mobilised from host to recipient by a helper plasmid such as pRK2013, also present in the host, which contains the RP4 transfer origin in a ColE1 replicon (and would therefore not be maintained in the recipient cell). The pRK290 recombinant would be contained as a single copy in the recipient cell, having no transfer origin or helper plasmid which would facilitate transfer to neighbouring cells.

It would have been possible to use a broad host range RP4 derived vector for library construction in *S. putrefaciens*, however a single stable mutation in the flavocytochrome c gene would have been relatively difficult to generate. Thus due to the availability of alternative strategies (Chapter 4 and further sections in this chapter) it was decided not to clone flavocytochrome c in *S. putrefaciens*. The remainder of this chapter describes the attempt to clone the complete flavocytochrome c gene by functional complementation of a characterised fumarate reductase mutation in *E. coli*.

5.2. Genomic library construction in pUN121.

MM294 was chosen as the host *E. coli* strain for construction and amplification of the library as in addition to its high transformation frequency, it will not restrict unmodified DNA and will methylate foreign DNA in subsequent replications. This was important in these circumstances, as the library, although constructed in a permissive host, was to be screened in a less permissive strain of *E. coli* which would restrict foreign, unmodified DNA. A consideration prior to library construction was the minimum genomic fragment size required for cloning into the vector to encode the full length flavocytochrome. From the flavocytochrome molecular weight of 84 kDa, it was estimated that a DNA sequence slightly larger than 2 kb would fully encode the flavocytochrome. Therefore for cloning by complementation, where expression of a functional flavocytochrome would demand a full gene and upstream promoter sequence, genomic fragments of 4-10 kb were selected for library construction. Chromosome was prepared and size fractionated as described in sections 4.1.-4.3.. Plasmid pUN121 (Figure 2.4.) was propagated in and isolated from the dam$^-$ *E. coli* strain NM547 to ensure that the *Bcl*I cloning site in the vector was unmethylated. Following ligation of 4-10 kb *Sau3A* chromosomal fragments to

the *Bcl*I digested plasmid and transformation into MM294, colonies harbouring recombinant plasmids were positively selected on agar containing both tetracycline and ampicillin and non-recombinant plasmids selected against being resistant only to ampicillin.

The number of independent transformants (N) required for complete representation of the *S. putrefaciens* chromosome was determined from the following equation according to Clarke and Carbon (1976).

$$N = \ln (1 - P)/\ln \{1 - [(L - X)/M]\}$$

P = the probability of finding the required clone in a library of N recombinants.
M = the genome size (in this case it was based on the *E. coli* genome size of 4.2 x 10^6 base pairs as the *S. putrefaciens* genome size has not been determined).
L = the average fragment size.
X = the size of fragment (gene) screened for.
The probability P was set at 99%, with the average size of a single cloned fragment of 7 kb previously estimated from the isolation of plasmid from ten randomly chosen independent pUN121 recombinants.
The number of independent transformants required with these parameters to give a 99% probability of including a sequence of DNA encoding the flavocytochrome was 3866. Approximately 4200 independent tetracycline resistant transformants were pooled to represent the library. (When N = 4200 in the above equation, P approaches 100%, indicating that the genome should be completely represented).

5.3. Library screening.

The library constructed and amplified in MM294 was transformed into JRG780 for screening. It was decided to screen for functional complementation of both tryptophan auxotrophy and fumarate reductase. The *trpA* gene is part of the tryptophan operon which is required for the synthesis of endogenous tryptophan. A mutation in this gene (as in *E. coli* JRG653 and JRG780) renders the host unable to synthesise tryptophan and thus incapable of growth on media which contained no tryptophan. The fumarate reductase mutation in JRG780 was isolated following nitrosoguanidine mutagenesis of the parental strain JRG653 (Lambden and Guest, 1976) as being unable to grow anaerobically with fumarate as a terminal electron acceptor. The mutation was in the A subunit of fumarate reductase, which encodes the catalytically active flavin-binding region. Complementation of this would restore the ability of JRG780 to grow anaerobically with fumarate as terminal electron acceptor.

To effectively screen the library, it was necessary to account for the lower growth temperature of *S. putrefaciens* relative to *E. coli* and a compromise sought which would give a reasonable growth rate of *E. coli* but would not be high enough to inhibit protein expression, or folding of mature protein from *S. putrefaciens*. A temperature for screening the library which was optimal for the growth of putative complementing clones was determined by plating JRG780 onto glycerol minimal medium at a density of 500 colony forming units (cfu) per plate (identical conditions for which the library was to be screened) which were then incubated at 37°C, 30°C, 28°C, 24°C, and 20°C until the colonies were fully grown (Table 5.3.). Colonies were estimated to be fully grown on this medium following 24 and 36 hours incubation at 37°C and 30°C respectively. The other temperatures, as expected, gave a much slower rate of growth, being 4-5 days before colonies were fully formed at 20°C and 24°C and 48 hours at

Table 5.3. Effect of temperature on growth rate of *E. coli* JRG 780 on glycerol minimal medium.

Incubation temperature	Incubation time (h) to obtain full colony size
37°C	24
30°C	36
28°C	48
24°C	96
20°C	120

JRG780 was plated on to glycerol minimal medium at a density of approximately 500 cfu per 7.5 cm diameter plate. Plates were incubated individually at 37°C, 30°C, 28°C, 24°C or 20°C until colonies were judged to be fully grown.

28°C. Incubation temperatures of 30°C and above were disregarded as being too high for the expression of functional protein from *S. putrefaciens* (under normal conditions this elevated temperature inhibits growth) and temperatures of less than 24°C regarded to be too low for efficient growth of *E. coli*. The optimum screening temperature was thus set at 28°C. Once the optimum incubation temperature was chosen, the effect of this temperature on tryptophan synthesis and fumarate reductase was investigated.

5.3.1. Effect of temperature on expression of tryptophan synthase.

This was difficult to assess, as both JRG653 and JRG780 hosted a mutant *trpA* subunit. Another strain of *E. coli*, MM294 was streaked out to obtain single colonies on tryptophan-containing glycerol minimal medium and glycerol minimal medium alone. Extent of growth on the two media of MM294 with respect to colony size size was compared by eye following 24 and 48 hours of incubation (Table 5.4.). There seemed to be no difference in the rate of growth and colony size in either the presence or absence of tryptophan in the media. JRG780 grew at the same rate on tryptophan-containing glycerol minimal medium as MM294 but as expected, growth was not supported on glycerol minimal medium alone.

5.3.2. Determination of the tryptophan spontaneous reversion frequency

The spontaneous reversion frequency was evaluated by plating 10,000 colonies of JRG780 at a density of 500 cfu/plate on to glycerol minimal medium lacking tryptophan (Table 5.5.). Following incubation, two spontaneous revertants were observed.

Table 5.4. Effect of temperature on expression of tryptophan synthase.

	tryptophan$^-$	tryptophan$^+$
MM294	+ + +	+ + +
JRG780	-	+ + +

MM294 (which is able to produce endogenous tryptophan) and JRG780 (*trpA*) were streaked out to achieve single colonies on glycerol minimal medium with and without tryptophan. Plates were incubated at 28^{o}C for 48 h following which the colony size and extent of growth were compared.

Table 5.5. Complementation of JRG 780 *trpA* by *S. putrefaciens* genomic library in pUN121.

	no. trp$^+$ colonies per 10^4 screened.
JRG780	2
JRG780 (pUN121 library)	3

JRG780 (*trpA*) and JRG780 (*trpA*) transformed with the *S. putrefaciens* genomic library constructed in pUN121, were screened for colonies with the ability to produce endogenous tryptophan at 28^{o}C for 48 h on glycerol minimal medium minus tryptophan at a density of 500 cfu per 7.5 cm plate.

5.3.3. Screen for trp$^+$ Recombinants.

Approximately 10,000 colonies from the pUN121 library in JRG780 were plated on to glycerol minimal medium minus tryptophan at a predetermined density of 500 cfu/ml. Following incubation, three colonies able to produce endogenous tryptophan grew (Table 5.5.). Although this was only one more colony than observed for spontaneous reversion, it was decided to investigate the ability of plasmid from any of these three colonies to complement the *trpA* mutation. The colonies were streaked separately on to L-agar containing tetracycline and ampicillin to confirm the presence of recombinant plasmid. Plasmid was then isolated from each colony, retransformed into a clean background of JRG780 and transformants selected on L-agar containing ampicillin and tetracycline. Individual retransformed colonies (20) hosting plasmid were streaked out to obtain a single line of growth on glycerol minimal medium minus tryptophan. This method was chosen for identification of putative complementing colonies, as streaking out for single colonies may have been confused with spontaneous revertants. None of the transformants harbouring the plasmid grew (a confluent line of growth corresponding to a plasmid complementing the *trpA* mutation) indicating that the putative complementing colonies on the initial screen resulted from spontaneous reversion and the *S. putrefaciens* DNA cloned on the plasmid was not responsible for complementation of the mutation.

5.3.4. Effect of temperature on expression of fumarate reductase

JRG653 and JRG780 were streaked out to give single colonies on glycerol fumarate minimal medium. Colonies of JRG653 were fully formed following 48 hours anaerobic incubation at 28^{o}C, therefore the incubation temperature seemed to have no adverse effects on the expression of fumarate

reductase (Table 5.6.). Very small colonies of JRG780 were observed, probably due to trace levels of oxygen in the agar diffusing slowly to the surface.

5.3.5. Determination of the fumarate reductase spontaneous reversion frequency

The spontaneous reversion frequency of fumarate reductase in JRG780 was determined in a similar way to that of *trpA*. Approximately 10,000 colonies of JRG780 were plated out at a density of 500 cfu/plate. No spontaneous revertants were observed after incubation at 28°C for 48 hours (Table 5.7.). Colonies of JRG653 plated out and incubated in parallel with JRG780 were fully grown, confirming that glycerol fumarate minimal medium was able to support anaerobic growth of colonies with a fully active fumarate reductase.

5.3.6. Screen for frd$^+$ Recombinants.

Approximately 10,000 pUN121 recombinants hosted in JRG780 were plated on to glycerol-fumarate minimal medium at a predetermined density of 500 cfu per plate. One putative positive colony was observed following incubation (Table 5.7.). This colony was plated on to L-agar containing ampicillin and tetracycline to confirm the presence of recombinant vector which was then isolated and retransformed into a clean background of JRG780. Transformants were selected on L-agar containing ampicillin and tetracycline and 10 single retransformed colonies were streaked out individually to single colonies on glycerol fumarate minimal medium. Streaking out to single colonies to determine whether growth resulted from functional complementation was chosen as a selective method as previously (5.4.5.) JRG780 had shown slight growth anaerobically on glycerol fumarate minimal medium. Following incubation, the plates of JRG653, JRG780 and JRG780 putative frd$^+$ colonies

Table 5.6. Effect of temperature on expression of fumarate reductase.

	fumarate$^-$	fumarate$^+$
JRG653	+ + +	+ + +
JRG780	+/-	+ + +

JRG653 (contains a fully functional fumarate reductase) and JRG780 (*frdA*) were streaked out to single colonies on glycerol fumarate minimal medium in the presence and absence of fumarate. Following incubation at 28^{o}C for 48 h, the colony size and extent of growth were compared.

Table 5.7. Complementation of JRG780 *frdA* by *S. putrefaciens* genomic library in pUN121.

	No. frd$^+$ colonies per 10^4 screened.
JRG780	0
JRG780 (pUN121 library)	1

JRG780, and JRG780 transformed with the *S. putrefaciens* genomic library constructed in pUN121, were plated on to glycerol minimal medium containing fumarate, at a colony density of 500 cfu per 7.5 cm plate. The plates were then incubated anaerobically for 48 h at 28^{o}C then examined for colonies able to utilise fumarate as a terminal electron acceptor.

were examined and comparisons of colony size made by eye. The putative Frd$^+$ colonies of JRG780 were smaller than those of the wild type JRG653 but did not seem to be quite as small as those of the mutant JRG780. As these results were rather inconclusive, to clarify the presence of any fumarate reductase activity, JRG653, JRG780, JRG780 hosting non-recombinant pUN121 and two of the ten original putative positive single colonies chosen randomly were cultured as in section 2.23.3 and the fumarate reductase activity measured and presented in Table 5.8. JRG653 as expected had the highest activity, approximately 40-fold greater than the others. JRG hosting non-recombinant pUN121 had an activity similar to that of JRG780 hosting recombinant plasmid. This indicated that the cloned fragment from *S. putrefaciens* had no complementing fumarate reductase activity. JRG780 alone had a slightly lower activity than JRG780 hosting either recombinant or non-recombinant plasmid, indicating that the plasmid had some slight enhancing effect on the anaerobic growth of JRG780.

In parallel with the enzyme assays, it was attempted to detect plasmid encoded protein corresponding to flavocytochrome c. No band development was observed. It was concluded that the original single colony able to utilise fumarate as a terminal electron acceptor was a spontaneous revertant. Although the original mutation was generated by nitrosoguanidine which results in a variety of mutations, a low spontaneous reversion frequency is possible depending on the type of mutation generated (Mandelstam, 1982).

5.4. Summary

No complementation of either tryptophan synthetase or fumarate reductase activity was achieved on transforming the pUN121 *S. putrefaciens* genomic library into JRG780 and selective screening. It was considered that the library was fully

Table 5.8. Determination of plasmid-encoded fumarate reductase activity.

Strain	Specific Activity*
JRG653	88 x 10^{-3}
JRG780	1.37 x 10^{-3}
JRG780 + pUN121	2.2 x 10^{-3}
JRG780 + pUN121 recombinant 1	2.2 x 10^{-3}
JRG780 + pUN121 recombinant 2	2.04 x 10^{-3}

* specific activity expressed as μ mol methyl viologen oxidised min^{-1} mg $protein^{-1}$.

Cultures were incubated aerobically in 60 ml L-broth to an A_{600} of 0.4. Glucose was added to 0.4% and fumarate to 0.04 M (final concentrations) and the flasks filled with sterile L-broth to create oxygen-limited growth conditions. Incubation was continued statically for 16 h, following which the fumarate reductase activity of each sample was measured.

representive of the entire genome (section 5.2.) and the screening conditions appropriate, as the temperature for screening the library although lower than the normal *E. coli* growth temperature, seemed not to inhibit the expression of either tryptophan synthase or fumarate reductase.

Tryptophan synthase is composed of two peptides which are gene products of *trpA* and *trpB* and catalyse the conversion of indoleglycerol-P to tryptophan. Prior to screening the library, it was considered that complementation of the *trpA* mutation had a reasonable chance of success as extensive studies and characterisation of the tryptophan synthase A subunit in various bacterial species had shown some interspecies homology.

Early comparative studies of the purified tryptophan synthase A subunit of several Enterobacteriaceae (*E. coli* K 12, *E. coli* B, *Shigella dysenteriae*, *S. typhimurium*, and *Aerobacter aerogenes*) indicated that they were of similar size, approximately 29 kDa (Creighton *et al*, 1966). The peptide patterns (following digestion with trypsin and chymotrypsin) of the two *E. coli* strains in this study were identical, with those from *S. typhimurium* and *A. aerogenes* less similar differing both from each other and from *E. coli*. However at least half of the peptides were common between any of the species studied which indicated some homologous regions on the protein. More detailed molecular analysis showed that the *trpA* of *E. coli* and *S. typhimurium* were 86% identical in amino acid sequence and 76% identical in nucleotide sequence (Nichols *et al*, 1979) with the ability of functional substitution to occur between both species indicating that the differences were in unimportant unconserved regions. Bacterial species which are not closely related to *E. coli*, such as the thermophile *T. thermophilus* (Kayama and Furokawa, 1990), was still able to functionally complement an *E. coli trpA* mutation despite the *T. thermophilus trpA* amino acid sequence having only 28.7% identity with *E. coli* and *S. typhimurium* and 42.6% identity with that

of *Bacillus stearothermophilus*. This indicated that even at low sequence similarities, conservation of functionally active sites on the protein allows complementation . The overall G+C ratio in *S. putrefaciens* at 44% differs markedly from those of the TrpA of both *E. coli* and *S. typhimurium* of 54% and 57.3% respectively (Nichols and Yanofsky, 1979) although that of *T. thermophilus* is much higher (70%) (Kayama and Furokawa, 1990) and codon usage not surprisingly quite different to that in *E. coli*. The TrpA subunit exhibits maximal enzyme activity only when complexed with the TrpB subunit, so functionally significant regions of the A subunit include not only the active site, but also the region involved in combining with the B subunit. If the *trpA* subunit from *S. putrefaciens* was unable to combine effectively with the *E. coli trpB* subunit then insufficient endogenous tryptophan would be produced to sustain growth on media lacking tryptophan.

Cloning flavocytochrome c by complementation of the *frdA* mutation in *E. coli* was not successful. The *frdA* encodes the flavin binding subunit of fumarate reductase in *E. coli* (Dickie and Weiner, 1979) which is the catalytically active site and bound to the cytoplasmic membrane via hydrophobic anchor proteins encoded by *frdC* and *frdD* (Lemire *et al*, 1982; Gundstrom and Jaurin, 1982), so as previously discussed, the *E. coli* fumarate reductase is different in structure and cellular location to flavocytochrome c. For the flavocytochrome to be catalytically active, then the haem and flavin prosthetic groups would have to be inserted into the apoprotein to allow the mature protein to assume correct conformation. Flavin incorporation usually occurs spontaneously eg. with flavocytochrome b_2 but attachment of c-type haems is enzymatic (Warren and Scott, 1990), and *E. coli* may not have enzymes that recognise flavocytochrome c.

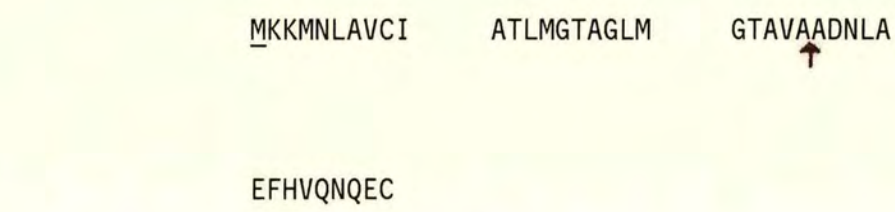
CHAPTER 6

FINAL DISCUSSION

A clone encoding a chromosomal fragment of approximately 1.5 kb was isolated from a *S. putrefaciens* genomic pEX3 expression library, giving a strong positive signal when developed by colony hybridization with antibody raised to purified flavocytochrome c. The protein expressed from this cloned fragment was not apparent as a single hybrid protein, but observed as several bands of 45 kDa and less (Plate 4.6). Furthermore these bands could only be detected by Western blots hybridising to flavocytochrome c antibody, and not by either silver or Kenacid blue staining following SDS PAGE. This suggested either (i) poor expression of protein from the cloned fragment, or (ii) instability of expressed protein within the host. The former explanation was considered unlikely as pEX3 is multicopy and has been reported to express protein from cloned DNA to a level constituting approximately 25% of total cell protein (Stanley, 1984). It was more likely that, as the library was screened in a host not disabled in native protease, such as *lon* (Mount, 1980), host protease activity contributed to the partial breakdown of expressed protein. Although the protein was difficult to size, being present as several bands, the largest band size of 45 kDa was not inconsistent with a protein encoded by a 1.5 kb DNA fragment. To size the fragment more accurately, the recombinant vector could have been transformed into a *lon* mutant of *E. coli* to minimise proteolysis. However it was considered that the most accurate method of sizing the fragment was to sequence it.

On sequencing the 1.5 kb cloned fragment, two non-overlapping segments (contigs) of 438 and 966 bp were identified. An in-frame continuous sequence with no stop codons was obtained on translation of each fragment (Figures 4.6 and 4.7) with the 438 bp contig preceding the 966 bp contig. A region of approximately 200 bp spanning the contigs remained unsequenced. Comparison of the predicted amino acid sequence of the contigs with the N-

Figure 6.1 Proposed flavocytochrome c leader sequence



↑ : signal peptidase cleavage site

M: computer-predicted N-terminal methionine

terminal sequence from purified flavocytochrome c protein identified a 8-residue sequence of identity , at the 5' terminus of the second, 322 residue (966 bp) contig. Homology of the first 8 N-terminal residues of the contig with N-terminal sequence of purified flavocytochrome c strongly indicated that this contig encoded the region of the flavocytochrome c protein which contained the N-terminus. No region of identity with other proteins was found for the other 146 residue (438 bp) contig within any of the databases. Recent attempts to sequence the unsequenced fragment between the two contigs (Reid and Manson, 1990, unpublished results) identified a sequence typical of a signal sequence, responsible for targeting the protein to the periplasm (Pugsley and Schwartz, 1985). The signal sequence typically featured a positively charged residue (lysine), followed by a stretch of hydrophobic and neutral residues before reaching the signal peptidase cleavage site. This strongly agreed with earlier biochemical studies relating to flavocytochrome c (Ward, unpublished results) which showed that it was located in the periplasm.

Further analysis of the 322 residues from the contig proposed to encode part of the flavocytochrome c structural gene, that was showed regions for both haem and flavin attachment. Four haem binding sites of the typical CXYCH motif were identified within the first 81 residues (Section 4.6). This region was subsequently designated the haem domain. No similarity was detected on comparison of this domain with other multihaem c-type cytochromes outwith the conserved CXYCH haem binding motif; neither was there any alignment similarity of the haem binding sites of flavocytochrome c and these other cytochromes. The second domain was designated as the flavin domain (Section 4.7) due to homology of a region from residues 123-151 of the flavocytochrome c sequence with an N-terminal FAD binding region common to many other flavoproteins (Figure 4.9). Similarity with other flavoproteins was confined to

this region with the exception of the succinate dehydrogenase of *E. coli* and *B. subtilis* with which there was approximately 30% sequence similarity over a region of 200 residues directly downstream of the FAD region.

Thus, it was proposed, supported by these findings, that this cloned fragment encoded part of the flavocytochrome c structural gene.

In parallel with cloning and sequencing the fragment in pEX3, it was attempted to isolate a recombinant encoding the complete flavocytochrome c structural gene, from pEX3 and pUN121 libraries, using the 1.5 kb cloned fragment, but no hybridising clones were identified. Attempts to clone the flavocytochrome c gene by genetic complementation of a fumarate reductase mutant of *E. coli* following transformation with a second *S. putrefaciens* gene library constructed in the positive selection vector pUN121 were also unsuccessful. At the time, it was thought that absence of complementation in *E. coli* (of both tryptophan synthase and fumarate reductase) probably indicated that *E. coli* and *S. putrefaciens* were genetically too diverse to allow functional complementation. However, the unsuccessful attempts to isolate a recombinant encoding the entire flavocytochrome c gene, from both pEX3 and pUN121 libraries using the 1.5 kb cloned fragment as an homologous probe suggested that neither library was fully representative of the entire *S. putrefaciens* genome.

For further studies it will be of paramount importance to have a complete sequence of flavocytochrome c. This will facilitate identification of active sites, such as the previously mentioned active site histidines (Section 4.5.2), important in the *B. subtilis* and *E. coli* fumarate reductases, and allow sequence comparison with other periplasmic cytochromes, and flavoproteins. Overall, it will promote a greater understanding of the function of flavocytochrome c.

Cloning the entire flavocytochrome c gene could be achieved by two relatively straightforward strategies based on information detailed in this thesis. The first would be to construct a *S. putrefaciens* genomic library of 1.5-2.5 kb *EcoRI* fragments (as Southern blotting of genome following complete digestion with restriction enzyme showed that almost the entire flavocytochrome c structural gene could be located on an *Eco*RI fragment of approximately 1.8 kb), which would then be screened with an *Eco*RI/*Hind*III probe constructed from the 1.5 kb cloned fragment. A second strategy to clone the full flavocytochrome c structural gene would be by polymerase chain reaction (PCR) amplification of the *Eco*RI fragment using primers for specific regions within this DNA sequence. As these primers would hybridise specifically to short sequences of the flavocytochrome c gene only this DNA would be amplified to any degree.

Alternatively, cloning flavocytochrome c could be attempted utilising the Omegon Km intersposon which has already been used in attempts to generate a single stable mutation in the flavocytochrome c gene (Perry, 1989; Green, 1990). In parallel with this, a genomic library could be constructed first restricting the *S. putrefaciens* chromosome with an enzyme such as *Bam*HI which has no site in the flavocytochrome c gene in a vector which was then conjugated into a flavocytochrome c fumarate reductase mutant of *S. putrefaciens*. The library would be screened for functional complementation of fumarate reductase. Suitable vectors for this library construction have been suggested in Sections 5.1.4, 1.13 and 1.14.

Obvious work relating to the function of flavocytochrome c would involve elucidation of the flavin and haem interactions within the holoprotein. This could be achieved by separate mutation of each haem and flavin binding sites, preventing prosthetic group attachment. The effect of each mutation on

enzyme activity would be assessed by measuring both fumarate and TMAO reductase activities. This will help us to understand how flavocytochrome c functions *in vitro*, both as a fumarate reductase and a TMAO reductase, by confirming which prosthetic groups are essential for either or both enzymic activities.

The mechanism of electron transfer between redox centres, eg haem and flavin, is at present under intensive research, particularly with respect to determining whether electron transfer occurs "through space" or "through bonds". Using artificial electron donors/acceptors to cytochrome c, Conrad *et al* (1991) concluded that "through bond" pathways were more important than "through space" distances. By contrast, Moser *et al* (1991) studying electron transfer in both photosynthetic reaction centres and in synthetic systems, concluded that intraprotein electron transfer is controlled by very few factors, with a direct relationship between electron transfer rate and distance between redox centres. Site directed mutagenesis has proved a powerful tool for testing such hypotheses. This approach has been used to show that intracomplex electron transfer between cytochrome c peroxidase and cytochrome c involves conformational changes (McLendon *et al*, 1991). Chapman and Reid (1991) have studied electron transfer in the pathway pyruvate --> flavin --> haem --> cytochrome c in yeast flavocytochrome b_2. Comparison of electron transfer rates between wild-type and mutants with altered residues close to the two redox centres enabled indentification of amino acids involved in catalytic electron transfer, and led to the proposal that a link region between the haem and flavin domains was important in bringing the redox centres close to each other. Such approaches would also be applicable to flavocytochrome c from *S. putrefaciens*.

Studies on the properties of c-type flavocytochromes such as those found in the phototrophic green and purple bacteria (Barsch, 1978; Sienmetz and Fischer, 1981; Fischer, 1988) and *Pseudomonas* spp (Hopper and Taylor, 1977) have shown that they are capable of oriented electron transfer in which electrons are moved from electron donor to acceptor via a defined path. Furthermore, these studies have offered the chance to understand in molecular terms the mechanisms and structure required for efficient intracomplex electron transfer.

If flavin is easily dissociated from the holoprotein by isoelectric focusing as in *Pseudomonas putida* (McIntire and Singer, 1982; Koerber *et al*, 1985), titration of the flavin with haem to yield a fully active reconstituted enzyme would allow evaluation of the dissociation constant in flavocytochrome c. Additionally, from flavin and haem titrations with oxidised subunits, reduced subunits and oxidised subunits in the presence of substrates or competitive inhibitors, the effect of the cytochrome subunit modulating the reactivity of the flavin subunit could be assessed. A clearer insight of the molecular interactions which result in these reactions would be resolved by a high resolution 3-D structure of the protein. As flavocytochrome c is soluble, like the flavocytochromes c of *Pseudomonas putida* and *Chromatium* it may be relatively easily crystallised.

Flavin interaction with individual haems might be studied following adduct formation. Phototrophic bacterial flavocytochrome c forms adducts between compounds such as sulphite, thiosulphate, cyanide or mercaptans and the flavin (Meyer and Bartsch, 1976), with flavin absorbance at 280 nm, 360 nm, 450 nm and 480 nm becoming bleached. The perturbance of a haem spin state, at a low pH, by flavin-adduct formation may indicate an interaction between flavin and haem. As flavocytochrome c from *S. putefaciens* has at least 2 haem

spin states it would be useful to use this technique to identify which interact with the flavin.

Flavin may also modulate the midpoint potential of haem in the holoprotein as observed in various *Pseudomonas* spp (Hopper, 1983) where the redox potential of the isolated haem subunit decreased to a level consistent with interaction between the flavin and haem subunits playing an important role in controlling the haem midpoint potential in the holoprotein. The mechanism of this modulation as yet is unknown, but may be investigated in more detail by comparison of the structure of the isolated haem subunit and the holoenzyme. In the flavocytochromes c of *Chlorobium* and *Chromatium* the important role of the protein-flavin and haem-flavin interactions in modulating the flavin environment was indicated by the finding that the flavin moiety redox potentials were much more positive than those of free flavins or typical flavoproteins (Cusanovich *et al*, 1985).

The study of electron transfer in flavocytochromes c have followed the principal approach of laser flash photolysis (Tollin *et al*, 1986; Cusanovich *et al*, 1988). This approach generates a free flavin semiquinone as a strong reductant, thus enabling the study of subsequent electron transfer between haem and flavin subunits of flavocytochrome c.

The flavin semiquinone dRFH rapidly reduced both haem and flavin moities of the *Pseudomonas putida* flavocytochrome c indicating that both redox centres were exposed at the enzyme surface (Bhattacharyya *et al*, 1985). Partial reduction of the haem and flavin subunits showed intra complex electron transfer with the neutral pseudomonad flavin semiquinone reducing the remaining oxidised haem. This result is in line with the assumption that substrate oxidation taking place on the flavin subunit would result in the formation of a neutral flavin semiquinone, with electrons subsequently

transferred to the haem subunit. Reduction kinetics of isolated haem and flavin subunits are the same in holoenzyme indicating that assembly of the subunits into the native complex does not appear to cause any steric hindrance to haem and flavin interaction domains (Bhattacharyya *et al*, 1985).

In contrast flavocytochromes c from *Chromatium* and *Chlorobium* are less reactive with dRFH suggesting steric hindrance to haem and flavin domains (Cusanovich *et al*, 1985). Adduct formation of endogenous flavin blocked haem reduction by both endogenous flavin seminquinones as well as fully reduced flavins indicating that as with *Pseudomonas putida*, flavocytochrome c sequential electron transfer proceeds from electron donor to the flavin subunit, followed by intra complex electron transfer to the haem subunit, and then reduction of the physiological electron acceptor. The techniques used to characterise these flavocytochrome c's could usefully be applied to flavocytochrome c.

The improved purification of flavocytochrome c (Chapter 3) will allow rapid isolation of sufficient protein for crystallisation studies. Flavocytochrome c protein was purified from periplasm isolated from cells of *S. putrefaciens* cultured microaerobically with fumarate as sole terminal electron acceptor, as flavocytochrome c was induced maximally during these conditions. The first purification yielded 4 mg purified flavocytochrome c from 10 litres of culture. Some proteolysis resulted following chromatography on hydroxyapatite, although this was prevented at following stages by addition of EDTA. Furthermore, gel filtration chromatography appeared to result in the loss of bound haem, although no haem was detected in fractions other than that corresponding to the single flavocytochrome c peak. The second purification had several advantages over the first (Section 3.4). It was more efficient requiring only three stages to purify the protein to homogeneity; there

appeared to be no loss of bound haem at any stage; and importantly very little proteolysis of flavocytochrome c protein was detected. In this thesis, purified flavocytochrome c was used for the production of antibody (primarily for library screening), and for analysis of flavocytochrome c fumarate reductase activity.

K_M values of 12.6 μM for fumarate and 357 μM for succinate combined with a correspondingly higher k_{cat} for fumarate reduction than succinate oxidation indicated that flavocytochrome c was very specific for fumarate and in addition was able to catalyse the oxidation of succinate although at a much slower rate (Section 3.6). Unusually and in contrast to the fumarate reductases of other Gram-negative bacteria, which are bound to the inner aspect of the cytoplasmic membrane (Lemire *et al*, 1982; Unden *et al*, 1980; Kroger *et al*, 1980), *S. putrefaciens* fumarate reductase activity was periplasmic. This appears to be a feature of the terminal reductases of *S. putrefaciens* as both nitrate and TMAO reductases were previously identified as also being periplasmic (Nasser, 1983, Clarke, 1984).

Periplasmic c-type cytochromes have an important role in electron transfer to and from periplasmic enzymes that oxidise substrates for growth or reduce electron acceptors (Ferguson, 1988). In periplasmic reductase reactions electrons must either originate from the periplasm or be moved from sites of substrate oxidation on the cytoplasmic side of the membrane (Ferguson and Page, 1990). Furthermore, electron flow to a water soluble periplasmic reductase (such as flavocytochrome c) can only be linked to a proton electrochemical gradient if electron flow between the two enzymes is catalysed by proton-translocating integral membrane proteins. In the case of flavocytochrome c (which acts as an intermediate electron donor in periplasmic TMAO reductase activity as well as a fumarate reductase) electrons are donated from cytoplasmic dehydrogenases such as formate and NADH. This

suggests that electron transfer to the periplasm, generating a proton-electrochemical gradient must be linked via an integral membrane protein.

Consumption of electrons coupled to a reduction reaction via an integral membrane protein results in the net movement of two protons from cytoplasm to periplasm. An example of this is electron transport from succinate to nitrous oxide in *P. denitrificans* (Ferguson, 1986), where the transfer of two electrons is associated with the net movement of two positive charges out of the cell and is thought to involve operation of a ubiquinone cycle.

Further work in this area would obviously include identification of integral membrane components which link electron flow from cytoplasmic dehydrogenases to flavocytochrome c. Additionally, it would be of interest to estimate the net yield of ATP from both TMAO and fumarate reduction.

Antibody raised to purified flavocytochrome c precipitated out fumarate reductase activity from both periplasm and purified flavocytochrome c in parallel (Figures 3.10 and 3.11). However, zymogram staining for fumarate reductase activity showed two zones of activity in the periplasmic sample (Plate 3.12). One corresponded to that of flavocytochrome c. The second was considered to be a partially denatured form of enzyme rather than another separate periplasmic fumarate reductase, as Western blotting of a periplasmic sample from cells grown on fumarate followed by development with flavocytochrome c specific IgG showed two protein bands which corresponded to those zones of zymogramic fumarate reductase activity (Plate 3.13). Only one protein band was observed on Western blotting of a periplasmic sample following SDS PAGE, which indicated complete protein denaturation by these conditions. Fumarate reductase activity was also observed on zymogram staining of flavocytochrome c following SDS PAGE, although the zone of activity was very faint. This was unexpected, as denaturing conditions would be

expected to remove the non-covalently bound FAD required for activity. The observed activity was considered to result from incomplete denaturation of the enzyme on native gel electrophoresis, followed by partial renaturation in the buffer preceding staining. To confirm this, *in-vitro* fumarate reductase assays of purified flavocytochrome c following denaturation with SDS and mercaptoethanol (both individually and combined) could be performed.

No evidence has been obtained to suggest the presence of more than one fumarate reductase in *S. putrefaciens* (Crowe, 1991, Ward unpublished results). Therefore flavocytochrome c would appear to be responsible for anaerobic fumarate reductase activity. An obvious next step in this research would be identification and characterisation of the succinate dehydrogenase in *S. putrefaciens*, aiming to show that it was a different enzyme from flavocytochrome c.

The discovery of a putative signal peptidase cleavage site proposed to be associated with flavocytochrome c is in line with current evidence from c-type cytochromes in *R. capsulatus* (Daldal *et al*, 1986), *R. sphaeroides* (Donohue *et al*, 1986) and *Desulfovibrio vulgaris* (Voordouw and Brenner, 1986), which established that the cytochrome-c polypeptide was synthesised with an N-terminal signal sequence. The sequence was typical of signal sequences that direct certain bacterial proteins to the periplasm, and indicated that bacterial c-type cytochromes (including flavocytochrome c) are exported to the periplasm through the cytoplasmic membrane. Haem incorporation, at least with mitochondrial cytochrome c, is associated with ordering of the polypeptide (Stellwagen *et al*, 1972), thus as translocation of the polypeptide requires it to be in an unfolded state, ordering and haem incorporation of c-type cytochromes would occur in the periplasm. Evidence supporting this has been obtained from a mutant of *P. denitrificans*, pleiotrophically deficient in periplasmic c-type

cytochromes, where the apo forms of the cytochromes are found in the periplasm (Page and Ferguson, 1989; 1990). Therefore, there must be a step involved in export of haem to the periplasm. This has been supported by studies on the expression of the *R. sphaeroides* cytochrome c_2 in *E. coli* (McEwan *et al*, 1989). This study showed that at least one of the components involved in haem export was anaerobically induced, and of low specificity, allowing catalysis of haem into a cytochrome not closely related to any found in *E. coli*.

In the case of flavocytochrome c, a flavin export system must be present in addition to haem export, assuming that flavin incorporation will occur in the periplasm with ordering of the protein. Testing of this hypothesis could be done in the first instance as by Page and Ferguson (1989; 1990) by generating mutants pleiotropically deficient in periplasmic fumarate or TMAO reductase activities. Periplasmic proteins could then be analysed for the apo-form of flavocytochrome c deficient in either flavin, haem or both.

The kinetic analysis and molecular sequence presented in this thesis supports the hypothesis that flavocytochrome c is a periplasmic fumarate reductase. This unusual protein therefore appears to play a dual role, as a terminal reductase during anaerobic and oxygen limited conditions, and as an electron carrier in the reduction of TMAO where it is reduced directly by formate. Further investigation of the flavocytochrome c sequence and structure will give a greater in-depth knowledge of the roles which this unusual and unique protein plays.

REFERENCES

Abraham, P.R., Boxed, D.H., Graham, A., Tucker, A.D., van't Riet, J. & Weintjes, F.B. (1981).
Immunochemical and structural comparison of the respiratory nitrate reductases from *Escherichia coli* and *Klebsiella aerogenes*.
FEMS Microbiological Letters **10**, 95-100.

Adams, M.W.W. & Mortenson, L.E. (1983).
The effect of cyanide and ferricyanide on the activity of the dissimilatory nitrate reductase of *Escherichia coli*.
Journal of Biological Chemistry **257**, 1791-1799.

Ambler, R. (1971).
The amino acid sequence of cytochrome c-551.5 (cyt c) from the green photosynthetic bacterium *Chloropseudomonas ethylica*.
FEBS Letters **18**, 351-353.

Ambler, R., Bruschi, M., LeGall, J. (1971).
The amino acid sequence of cytochrome c_3 from *Desulfovibrio desulfuricans* (strain I Agheuilla NCIB 8380)
FEBS Letters **18**, 347-350.

Amy, N.K. & Rajagopalan, K.U. (1979).
Characterisation of molybdenum cofactor in *Escherichia coli*.
Journal of Bacteriology **140**, 114-124.

Anraku, Y. & Gennis, R.B. (1987).
The aerobic respiratory chains of *Escherichia coli*.
Trends in Biochemical Sciences **12**, 262-266.

Azoulay, E.J., Puig, J. & Pichinoty, F. (1967).
Alteration of respiratory particles by mutation in *Escherichia coli* K-12.
Biochemical and Biophysical Research Communications **27**, 270-274.

Bagg, A. & Nielands, J.B. (1987).
Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*.
Biochemistry **25**, 5471-5477.

Baker, T., Grossman, A. & Gross, C. (1980).
A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis.
Proceedings of the National Academy of Science USA **81**, 6779.

Ballard, A.L. & Ferguson, S.J. (1987).
Molecular properties of the respiratory nitrate reductase of *Paracoccus denitrificans*.
Biochemical Society Transactions **15**, 937-938.

Barlow, D.J., Edwards, M.S. & Thornton, J.M. (1986).
Continuous and discontinuous protein antigentic determinants.
Nature **322**, 747-748.

Barrett, E.L. & Riggs, D.L. (1982).
Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimurium*.
Journal of Bacteriology **150**, 563-571.

Bartsch, R.G. (1978).
Cytochromes, *In* The Photosynthetic Bacteria (Clayton, R.K. & Sistrom, W.R. eds), Plenum Press, New York. 249.

Barth, P.T. and Grinter, N.J. (1974).
Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring limited resistance to streptomycin and sulfonamides.
Journal of Bacteriology **120**, 618-630.

Batut, J., Daveran-Mingot, M.-L., David, M., Jacobs, J., Gamerone, A.M. & Kahn, D. (1989).
fixK, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*.
EMBO Journal **8**, 1279-1286.

Beatty, S.A. & Gibbons, N.E. (1937).
The measurement of spoilage in fish.
Journal of the Biological Board of Canada **3**, 77-91.

Benjamin, D.C., Berzofsy, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E. & Wilson, A.C. (1984).
The antigenic structure of proteins: A reappraisal.
Annual Review of Immunology **2**, 67-101.

Bergstrom, S., Lindberg, F.P., Olsson, O. & Normark, S. (1983).
Comparison of the overlapping frd and ampC operons of *Escherichia coli* with the corresponding DNA sequences in other gram-negative bacteria.
Journal of Bacteriology **155**, 1297-1305.

Bhattacharyya, S., Tollin, G., McIntire, W. & Singer, T.P. (1985).
Laser-flash-photolysis studies of *p*-cresol methyl hydroxylase.
Biochemical Journal **228**, 337-345.

Bilous, P.T. & Weiner, J.H. (1988).
Molecular cloning and expression of the *Escherichia coli* dimethyl sulfoxide reductase operon.
Journal of Bacteriology **170**, 1511-1518.

Birdsell, D.C. & Cota-Robles, E.M. (1967).
Production and ultrastructure of lysozyme and EDTA-lysozyme spheroplasts of *Escherichia coli*.
Journal of Bacteriology **93**, 427 - 437.

Birnboim, H.C. & Doily, J. (1979).
A rapid alkaline extraction procedure for screening recombinant plasmid DNA.
Nucleic Acids Research **7**, 1513-1523.

Blattner, F.R., Williams, B.G., Blechl, A.E., Deniston-Thompson, K., Faber, H.E., Furlong, L.A., Grunwald, D.J., Kiefer, D.O., Moore, D.D., Schuman, J.W., Sheldon, E.L. & Smithies, O. (1977).
Charon phages: safer derivatives of bacteriophage lambda for DNA cloning.
Science **196**, 161-169.

Bolivar, F. (1978).
Construction and characterisation of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *EcoRI* sites for selectionof *EcoRI* generated recombinant DNA molecules.
Gene **4**, 121-136.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.V., Heynecker, H.L., Boyer, H.W., Crosa, J.H. & Falkow, S. (1977).
Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system.
Gene **2**, 95-113.

Boonstra, J., Huttunen, M.T., Konings, W.N. & Kabak, H.R. (1975).
Anaerobic transport in *Escherichia coli* membrane vesicles.
Journal of Biological Chemistry **250**, 6792-6798.

Boulnois, G.J. (1987).
Gene cloning and analysis, a laboratory guide.
Blackwell Scientific Publications.

Boulnois, G.J., Varley, J.M., Sharpe, G.S. & Franklin, F.C.H. (1985).
Transposon donor plasmids based on ColIb-P9, for use in *Pseudomonas putida* and a variety of other Gram negative bacteria. Molecular and General Genetics **200**, 65-67.

Boxer, D.H. & Clegg, R.A. (1975).
A trans-membrane location for the proton translocating reduced ubiquinone-nitrate reductase segment of the respiratory chain of *Escherichia coli*.
FEBS Letters **60**, 54-57.

Bragg, P.D. & Hackett, N.R. (1983).
Cytochromes of trimethylamine N-oxide anaerobic respiratory pathway of *Escherichia coli*.
Biochimica et Biophysica Acta **725**, 168-177.

Brenner, S., Cesarni, G. & Kan, J. (1982).
Phasmids: hybrids between Co1E1 plasmids and *Escherichia coli* bacteriophage lambda.
Gene **17**, 27-44.

Broome, S. & Gilbert, W. (1978).
Immunological screening method to detect specific translation products.
Proceedings of the National Academy of Science USA **75**, 2746-2749.

Brown, T.D., Jones-Mortimer, M.C. & Kornberg, H.L. (1977).
The enzymatic interconversion of acetate and acetyl-Co-A in *Escherichia coli*.
Journal of General Microbiology **102**, 327-336.

Busby, S.J.W. (1986).
Positive regulation in gene expression.
Symposium of the Society for General Microbiology **39**, 51-77.

Cammack, R., Pahl, D.S. & Weiner, J.H. (1980).
Evidence that centre 2 in *Escherichia coli* fumarate reductase is a [4Fe-4S] cluster.
Biochimica et Biophysica Acta **870**, 538-544.

Campbell, M. (1986)
Characterisation of cryptic plasmids from *Alteromonas* spp.
Honours thesis, Edinburgh University Department of Microbiology

Carlson, C.A., Ferguson, L.P. and Ingraham, J.L. (1982).
Properties of dissimilatory nitrate reductase purified from the denitrifier *Pseudomonas aeruginosa*.
Journal of Bacteriology **151**, 162-171.

Ceccini, G., Ackrell, B.A.C., Deshler, J.O. & Gunsalus, R.P. (1985).
Reconstitution of quinone reduction and characterisation of *Escherichia coli* fumarate reductase activity.
The Journal of Biological Chemistry **261**, 1808-1814.

Chassy, B.M. & Flickinger, J.L. (1987).
Transformation of *Lactobacillus casei* by electroporation.
FEMS Microbiology Letters **44**, 173.

Chaudhry, G.R. & MacGregor, C.H. (1983).
Cytochrome *b* from *Escherichia coli* nitrate reductase. Its properties and association with the enzyme complex.
Journal of Biological Chemistry **258**, 5819-5827.

Chippaux, M., Bonnefoy-Orth, V., Ratouchniak, J. & Pascal, M.C. (1981).
Operon fusions in the nitrate reductase operon and study of the control gene *nirR* in *Escherichia coli*.
Molecular and General Genetics **182**, 477-479.

Chippaux, M., Guidici, D., Abou-Jaeude, A., Casse, F. & Pascal, M.-C. (1978).
A mutation leading to total lack of nitrate reductase activity in *Escherichia coli* K12.
Molecular and General Genetics **160**, 225-229.

Clark, A.J. & Warren, G.J. (1979).
Conjugal transmission of plasmids.
Annual Review of Genetics **13**, 99-125.

Clark, D. & Cronan, J.E. (1980).
Escherichia coli mutants with altered control of alcohol dehydrogenase and nitrate reductase.
Journal of Bacteriology **141**, 177-183.

Clarke, G.J. (1984).
The purification and properties of trimethylamine N-oxide reductase from Alteromonas sp NCMB 400.
PhD thesis, Edinburgh University.

Clarke, G.J. & Ward, F.B. (1987). Purification and properties of trimethylamine N-oxide reductase from *Shewanella* sp. NCMB 400.
Journal of General Microbiology **133**, 379-386.

Clarke, L. & Carbon, J. (1976).
A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome.
Cell **9**, 91-99.

Clayton, R.C., Sistron, W. R., eds (1978).
The Photosynthetic Bacteria. NY Plenum, 946pp.

Cohen, S.N., Chang, A.C.Y. & Hsu, L. (1972).
Non chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA.
Proceedings of the National Academy of Science USA **69**, 2110-2114.

Cole, J.A. & Ferguson, S.J. (1988).
The Nitrogen and Sulphur Cycles.
Symposia of the Society for General Microbiology **42**.

Cole, J.A. & Ward, F.B. (1973).
Nitrate reductase-deficient mutants of *Escherichia coli* K12.
Journal of General Microbiology **76**, 21-29.

Cole, S.T. (1982).
Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*.
European Journal of Biochemistry **122**, 479-484.

Cole, S.T., Condon, C., Lemire, B.D. & Weiner, J.H. (1985).
Molecular biology, biochemistry and bioenergetics of fumarate reductase, a complex membrane-bound iron-sulphur flavoenzyme.
Biochimica et Bio physica Acta **811**, 381-403.

Cole, S.T., Gundstrom, T., Jaurin, B., Robinson, J.J. & Weiner, J.H. (1982).
Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulphur protein subunit of the fumarate reductase of *Escherichia coli*.
European Journal of Biochemistry **126**, 211-216.

Coleman, K.J., Cornish-Bowden, A. & Cole, J.A. (1978).
Purification and properties of nitrite reductase from *Escherichia coli* K-12.
Biochemical Journal *175*, 483-493.

Collins, J. & Hohn, B. (1979). Cosmids: a type of plasmid gene-cloning vector that is packageable *in vitro* in bacteriophage λ heads.
Proceedings of the National Academy of Science USA **80**, 278-282.

Colonna-Ramano, S., Arnold, W., Shulter, A., Boistard, P., Puhler, A. & Priefer, U.B. (1990).
Rhizobium leguminosarum FnrN shows structural and functional homology to *Rhizobium meliloti* FixK.
Molecular and General Genetics **223**, 138-147.

Conrad, D.W., Chan, M.L., Corren, A., Eidsness, M.K. & Scott, R.A. (1991).
Long-range intramolecular electron transfer in cytochrome *c* derivatives containing covalently attached transition metal complexes.
Journal of Inorganic Biochemistry **43**, 88.

Cox, J.C. & Knight, R. (1981). Trimethylamine N-oxide (TMAO) reductase activity in chlorate resistant or respiration-deficient mutants of *Escherichia coli*.
FEMS Microbiology Letters **12**, 249-252.

Craske, A. & Ferguson, S.J. (1986).
The respiratory nitrate reductase from *Paracoccus denitrificans*. Molecular characterisation and kinetic properties.
European Journal of Biochemistry **158**, 429-436.

Creighton, T.G., Helinski, D.R., Somerville, R.L., Yanofsky, C. (1966).
Comparison of the tryptophan synthetase α subunits of several species of Enterobacteriacea.
Journal of Bacteriology **91**, 1819-1826.

Crowe, C. (1991).
Fumarate reductase activity in *Shewanella putrefaciens* NCMB400.
Honours thesis, Edinburgh University Department of Microbiology

Cusanovich, M.A., Meyer, T.E. & Tollin, G. (1985).
Flavocytochromes *c*: Transient kinetics of photoreduction by flavin analogues.
Biochemistry **24**, 1281-1287.

Cusanovich, M.A., Hazzard, J.T., Meyer, T.E. & Tollin, G. (1988).
Intramolecular and intracomplete electron transfer in redox proteins.
In Oxidases and Related Redox Systems, King, T.E., Mason, H.S. & Morrison, M. (eds). Alan Liss, New York. p401.

Daldal, F., Cheng, S., Applebaum, J., Davidson, E. & Prince, R.C. (1980).
Cytochrome c_2 is not essential for photosynthetic growth of *Rhodopseudomonas capsulata*.
Proceedings of the National Academy of Science USA **83**, 2012-2016.

Davis, R.W., Botstein, D. & Roth, J.R. (1980).
Advanced Bacterial Genetics.
A Manual for Genetic Engineering.

De Crombrugghe, B., Busby, S. & Buc, H. (1984).
Cyclic AMP receptor protein: role in transcription activation.
Science **224**, 831-838.

Dente, L. (1983).
pEMBL: a new family of single stranded plasmids.
Nucleic Acid Research **11**, 1645-1655.

De Wet, J.R., Fukushima, H., Dewji, N.N., Wilcox, E., O'Brien, J.S. & Helsinski, D.R. (1984).
Chromogen immune detection of human serum albumin and α-L-fucosidase clones in a human hepatoma cDNA expression library.
DNA **3**, 437.

Dickie, P. & Weiner, J.H. (1979).
Purification and characterisation of membrane-bound fumarate reductase from anaerobically grown *Escherichia coli*.
Canadian Journal of Biochemistry **57**, 813-821.

Dimroth, P. (1987).
Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria.
Microbiological Reviews **51**, 320-360.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D.P. (1980).
Broad host range DNA cloning system for Gram-negative bacteria. Construction of a gene bank of *Rhizobium meliloti*.
Proceedings of the National Academy of Science USA **77**, 7347-7351.

Donohue, T.J., McEwan, A.G. & Kaplan, S. (1986).
Cloning DNA sequence and expression of the *Rhodobacter sphaeroides* cytochrome c_2 gene.
Journal of Bacteriology **168**, 962-972.

Drews, G. (1985).
Structure and functional organisation of light harvesting complexes and photochemical reaction centres in membranes of phototrophic bacteria.
Microbiological Reviews **49**, 59-70.

Dubourdieu, M., Andrade, E. & Puig, J. (1976).
Molybdenum and chlorate resistant mutants in *Escherichia coli* K-12.
Biochemical and Biophysical Research Communications **70**, 766-773.

Easter, M.C. (1982).
Trimethylamine N - oxide reduction by *Alteromonas* spp.
PhD thesis. Torry Research Station, Aberdeen and Robert Gordon's Institute of Technology, Aberdeen, Scotland.

Easter, M.C., Gibson, D.M. & Ward, F.B. (1983).
The induction and location of trimethylamine-n-oxide reductase in *Alteromonas* sp. NCMB 400.
Journal of General Microbiology **129**, 3689-3696.

Ehrich, E., Craig, A., Poustka, A., Frischauf, A.-M. & Lehrach, H. (1987).
A family of cosmid vectors with the multi-copy R6K replication origin.
Gene **57**, 229-237.

Ehrlich, S.D., Cohen, S.N. & McDevitt, H.G. (1987).
A sensitive radioimmunoassay for detecting products translated from cloned DNA fragments.
Cell **13**, 681-689.

Eiglmeier, K., Honore, N., Iuchi, S., Lin, E.C.C. & Cole, S.T. (1989).
Molecular genetic analysis of Fnr dependent promoters.
Molecular Microbiology **3**, 869-879.

Ells, A.H. (1959).
A colourimetric method for the assay of soluble succinic dehydrogenase and pyridine nucleotide-linked dehydrogenases.
Archives of Biochemistry and Biophysics **85**, 561-562.

Enoch, H.G. & Lester, R.L. (1974).
The role of a novel cytochrome b-containing nitrate reductase and quinone in the *in vitro* reconstitution of formate-nitrate reductase activity of *Escherichia coli*.
Biochemical and Biophysical Research Communications **61**, 1234-1241.

Enoch, H.G. & Lester, R.L. (1975).
The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*.
Journal of Biological Chemistry **250**, 6693-6705.

Eventoff, W., Rossman, M.G., Taylor, S.S., Torff, H.-J., Meyer, H., Kiet, W. & K)iltz, H.-H. (1977).
Structural adaptations of lactate dehydrogenase isoenzymes.
Proceedings of the National Academy of Science USA **74**, 2766-2681.

Fellay, R., Krisch, H.M., Prentki, P. & Frey, J. (1989).
Omegon-Km: a transposable element designed for in vivo insertional mutagenesis and cloning of genes in Gram-negative bacteria.
Gene **76**, 215-226.

Ferguson, S.J. (1986).
The ups and downs of P\O ratios (and the question of non-integral coupling stochiometries for oxidative phosphorylation and related processes).
Trends in Biochemical Sciences **11**, 351-353.

Ferguson, S.J. (1988).
Periplasmic electron transport.
In Bacterial Energy Transduction (Anthony, C. ed), pp293-316. Academic Press, London.

Ferguson, S.J. & Page, M.D. (1990).
The functions and biosynthesis of the c-type cytochromes of bacterial respiratory chains with particular reference to autotrophs and the handling of C_1 compounds.
FEMS Microbiology Reviews **87**, 227-234.

Fischer, U. (1988).
Soluble electron transfer proteins of Chlorobiacaea, *In* Green Photosynthetic Bacteria (Olson, J.M., Ormensd, J.G., Amesz, J., Stackebrandt, E. & Truper, H.G. eds), Plenum Press, New York. 127.

Fox, T.D. (1987).
Natural variation in the genetic code.
Annual Review of Genetics **21**, 67-91.

Fukumori, Y. & Yamanaka, T. (1979).
Flavocytochrome c of *Chromatium vinosum*.
Journal of Biochemistry **85**, 1405.

Gamp, H., Maeder, M. & Zuberbuhler, A.D. (1980).
Standard test squares method.
Talanta **27**, 513-518.

Gershoni, J.M. & Palade, G.E. (1983).
Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to a positively charged membrane filter.
Analytical Biochemistry **124**, 396-405.

Gibson, D.H. (1981).
R-factor transfer in a marine pseudomonad.
Honours thesis, Edinburgh University Microbiology Department.

Glaser, J.H. & DeMoss, J.A. (1971).
Pleiotropic restoration by molybdate of nitrate reductase activity in *chlD* mutants of *Escherichia coli*.
Journal of Bacteriology **108**, 854-860.

Gorbunoff, M.J. (1984a).
The interaction of proteins with hydroxyapatite. I. Role of protein charge and structure.
Analytical Biochemistry **136**, 425-432.

Gorbunoff, M.J. (1984b)
The interaction of proteins with hydroxyapatite. II. Role of acidic and basic groups.
Analytical Biochemistry **136**, 433-439.

Gorbunoff, M.J. & Timasheff, S.N. (1984).
The interaction of proteins with hydroxyapatite. III. Mechanism.
Analytical Biochemistry **136**, 440-445.

Gouy, M. & Gautier, C. (1982).
Codon usage in bacteria. Correlation with gene expressivity.
Nucleic Acids Research **10**, 7055-7074.

Graham, A. & Boxer, D.H. (1980).
Arrangement of respiratory nitrate reductase in the cytoplasmic membrane of *Escherichia coli*.
Location of β subunit.
FEBS Letters **113**, 15-20.

Grassi, H.C., Andrade, E. & Puig, J. (1979).
Nitrate reductase and the membrane composition of pleiotropic chlorate resistant mutants of *Escherichia coli* K-12.
Biochemical and Biophysical Research Communications **90**, 86-91.

Gray, G.O. & Knaff, D.B. (1982).
The role of a cytochrome c-552-cytochrome *c* complex in the oxidation of sulfide in *Chromatium vinosum*.
Biochimica et Biophysica Acta **680**, 290-296.

Green, A.A. & Hughs, W.L. (1955).
Ammonium sulphate precipitation of protein.
Methods in Enzymology **1**, 67.

Green, B. (1990).
Isolation of fumarate reductase mutants of *Shewanella putrefaciens*.
Honours thesis, Edinburgh University Microbiology Department.

Grillet, L. & Giordono, G. (1983).
Identification and purification of a protein involved in the activation of nitrate reductase in the soluble fraction of a *chlA* mutant of *Escherichia coli* K12.
Biochimica et Biophysical Acta **749**, 115-124.

Grosjean, J. & Fiers, W. (1982).
Preferential codon usage in prokaryotic genes. The optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes.
Gene **18**, 199-209.

Grossin, G., Johnson, A., Pabo, C. & Sauer, R. (1983).
Repressor and Co protein: Structure, function and role in lysogenisation. *In* "Lambda II" (R.W. Hendrix, J.W. Roberts, F.W. Stahl & R. A. Weisberg eds), pp93-121. Cold Spring Harbor Monograph, Cold Spring Harbor, NY.

Grunstein, M. & Hogness, D.S. (1975).
Colony hybridisation: a method for the isolation of cloned DNAs that contain a specific gene.
Proceedings of the National Academy of Science USA **72**, 3961-3965.

Gundstrom, T. & Jaurin, B. (1982).
Overlap between *amp*C and frd operons on the *Escherichia coli* chromosome.
Proceedings of the National Academy of Science USA **79**, 1111-1115.

Hackett, N.R. & Bragg, P.D. (1983).
The association of two distinct *b* cytochromes with the respiratory TMAO reductase of *Escherichia coli*.
FEMS Microbiology Letters **13**, 213-217.

Hanahan, D. & Meselson, M. (1980).
Plasmid screening at high colony density.
Gene **10**, 63-67.

Harlander, S.K. (1987).
Transformation of *Streptococcus lactis* by electroporation.
In Streptococcal genetics, eds J. Ferreti & R. Curtiss, III pgs 229. American Society for Microbiology, Washington DC.

He, S.H., Der Vertanian, D.V. & LeGall, J. (1986).
Isolation of a fumarate reductase from *Desulfovibrio mutispirans*, a sulphate reducing bacterium.
Biochemical and Biophysical Research Communications **135**, 1000-1007.

Hederstedt, L. & Hatefi, Y. (1986).
Modification of bovine heart succinate dehydrogenase with ethoryformic anhydride and rose bengal: evidence for essential histidyl residues protectable by substrates.
Archives of Biochemistry and Biophysics **247**, 346-354.

Helfman, D.M., Feramisco, J.R., Fiddes, J.R., Thomas, G.P. & Hughes, S.H. (1983).
Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library.
Proceedings of the National Academy of Science USA **80**, 31-35.

Hellingwerf, K.J., Bolscher, J.G.M. & Konings, W.N. (1981).
The electrochemical proton gradient generated by the fumarate-reductase system in *Escherichia coli* and its bioenergetic implications.
European Journal of Biochemistry **113**, 369-374.

Henderson, N., Austin, S. & Dixon, R.A. (1989).
Role of metal ions in negative regulation of nitrogen fixation by the *nifL* gene product from *Klebsiella pneumoniae*.
Molecular and General Genetics **216**, 484-491.

Hirsch, C.A., Raminsky, M., Davis, B.D. & Lin, E.C.C. (1963).
A fumarate reductase in *E.coli* distinct from succinate dehydrogenase.
Journal of Biological Chemistry **238**, 3770-3774.

Hopper, D.J. (1983).
Redox potential of the cytochrome *c* in the flavocytochrome p-cresol methylhydroxylase.
FEBS Letters **161**, 100-102.

Hopper, D.J. & Taylor, D.G. (1977).
The purification and properties of p-cresol-(acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Pseudomonas putida*.
Biochemical Journal **167**, 155-162.

Ingledew, W.J. (1983).
The electron transport chain of *Escherichia coli* grown anaerobically with fumarate as terminal electron acceptor: an electron paramagnetic resonance study.
Journal of General Microbiology **129**, 1651-1659.

Ingledew, W.J. & Poole, R.K. (1984)
The respiratory chains of *Escherichia coli*.
Microbiological Reviews **48**, 222-271.

Iuchi, S., & Lin, E.C.C. (1987).
Moybdenum effector of fumarate reductase repression and nitrate reductase induction in *Escherichia coli*.
Journal of Bacteriology **169**, 3720-3725.

Iuchi, S. & Lin, E.C.C. (1988).
arcA (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways.
Proceedings of the National Academy of Science USA **85**, 1888-1892.

Iuchi, S. & Lin, E.C.C. (1989).
A second global regulator gene (arcB) mediating repression of enzymes in aerobic pathways of *Escherichia coli*.
Journal of Bacteriology **171**, 868-873.

Iuchi, S., Matsuda, Z., Fujiwara, T. & Lin, E.C.C. (1990a).
The *arcB* gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the arc modulon.
Molecular Biology **4**, 715-727.

Iuchi, S., Chepuri, V., Fu, M.-A., Gennis, R.B. & Lin, E.C.C. (1990b).
Requirement for terminal cytochromes in generation of the aerobic signal for the *arc* regulatory system in *Escherichia coli*: study utilising deletions and *lac* fusions of *cyo* and *cyd*.
Journal of Bacteriology **172**, 6020-6025.

Jamieson, D.J. & Higgins, C.F. (1984).
Anaerobic and leucine-dependent expression of a peptide transport gene in *Salmonella typhimurium*.
Journal of Bacteriology **160**, 131-136.

Jamieson, D.J., Sawers, R.G., Rugman, P.A., Boxer, D.H. & Higgins, C.F. (1986).
Effects of anaerobic regulatory mutations and catabolite repression on regulation of hydrogen metabolism and hydrogenase isoenzyme composition in *Salmonella typhimurium*.
Journal of Bacteriology **168**, 405-411.

Jayaraman, P.-S., Cole, J.A. & Busby, S.J.W. (1989).
Mutational analysis of sequences at the Fnr-dependent *nirB* promoter in *Escherichia coli*.
Nucelic Acids Research **17**, 135-145.

Jeter, R.M., Sias, S.R. & Ingraham, J.L. (1984).
Chromosomal location and function of genes affecting *Pseudomonas aeruginosa* nitrate assimilation.
Journal of Bacteriology **157**, 673-677.

Johnson, M.E. & Rajagopalan, K.U. (1987).
Involvement of *chlA, E, M* and *N* loci in *Escherichia coli* molybdoprotein synthesis.
Journal of Bacteriology **169**, 117-125.

Johnson, M.K., Bennett, D.E., Morningstar, J.G., Adams, M.W.W. & Mortensen, L. E. (1985).
The iron-sulfur cluster composition of *Escherichia coli* nitrate reductase.
Journal of Biological Chemistry **260**, 5456-5463.

Jones, H.M. & Gunsalus, R.P. (1985).
Transcription of the *Escherichia coli* fumarate reductase genes (frdABCD) and their co ordinate regulation by oxygen, nitrate and fumarate.
Journal of Bacteriology **164**, 1100-1109.

Jones, H.M. & Gunsalus, R.P. (1987).
Regulation of *Escherichia coli* fumarate reductase (frd ABCD) operon expression by respiratory electron acceptors and the fnr gene product.
Journal of Bacteriology **169**, 3340-3349.

Kahn, P.H., Powell, J.F., Beaumont, A., Roques, B.P. & Mallet, J.J.B. (1987).
An antibody purified with a λgt11 fusion protein precipitates enkephalinase activity.
Biochemical and Biophysical Research Communications **145**, 488-493.

Kalman, L.V. & Gunsalus, R.P. (1988).
The frdR gene of *Escherichia coli* globally regulates several operons involved in anaerobic growth in response to nitrate.
Journal of Bacteriology **170**, 623-629.

Kalman, L.V. & Gunsalus, R.P. (1989).
Identification of a second gene involved in global regulation of fumarate reductase and other nitrate-controlled genes for anaerobic respiration in *Escherichia coli*.
Journal of Bacteriology **171**, 3810-3816.

Kayama, Y. & Furokawa, K. (1990).
Cloning and sequence analysis of tryptophan synthatase genes of an extreme thermophile, *Thermus thermophilus* HB27. Plasmid transfer from replica-plated *Escherichia coli* recombinant colonies to competent *T. theromphilus* cells.
Journal of Bacteriology **172**, 3490-3495.

Kemp, D.J. & Cowman, A.F. (1983).
Direct immunoassay for detecting *Escherichia coli* peptides encoded by cloned DNA segments.
Proceedings of the National Academy of Science USA **78**, 4520-4524.

Kenney, W.C., McIntire, W. & Yananaka, T. (1977).
Structure of the covalently bound flavin of *Chlorobium* cytochrome c_{553}.
Biochimica et Biophysica Acta **483**, 467-474.

Kenney, W.C., Mowey, P.C., Seng, L.R. & Stogo, T.P. (1977).
Localisation of the substrate and oxaloacetate binding site of succinate dehydrogenase.
Journal of Biological Chemistry **251**, 2369-2373.

Kita, K., Konishi, K. & Anraku, Y. (1984).
Terminal oxidases of *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome b_{558}-d complex from cells grown with limited oxygen and evidence of branched electron-carrying systems.
Journal of Biological Chemistry **259**, 3375-3381.

Kleckner, N. (1981).
Transposable elements in prokaryotes.
Annual Review of Genetics **15**, 341-404.

Koerber, S.C., McIntire, W., Bohmont, C. & Singer, T.P. (1985).
Resolution of the flavocytochrome *p*-cresol methylhydroxylase into subunits and reconstitution of the enzyme.
Biochemistry **24**, 5276-5280.

Konings, W.N. & Kaback, H.R. (1973).
Anaerobic transport in *Escherichia coli* membrane vesicles.
Proceedings of the National Academy of Science USA **70**, 3370-3381.

Kowalski, J., Smith, J.H., Ng, N. & Denhardt, D.T. (1985).
Vectors for the direct selection of cDNA clones corresponding to mammalian cell mRNA of low abundance.
Gene **35**, 45-54.

Kranz, R.G. & Gennis, R.B. (1984). Characterisation of the cytochrome *d* terminal oxidase of *Escherichia coli* using polyclonal and monoclonal antibodies.
Journal of Biological Chemistry **259**,7998-8003.

Kroger, A., Dorrer, E. & Winkler, E. (1980).
The orientation of the substrate sites of formate dehydrogenase and fumarate reductase in the membrane of *Vibrio succinogenes*.
Biochimica et Biophysica Acta **589**, 118-136.

Kroger, A. & Innerhoffer, A. (1976).
The function of menaquinone, covalently bound FAD and iron sulphur protein in the electron transport from formate to fumarate of *Vibrio succinogenes*.
European Journal of Biochemistry **69**, 487-495.

Krulwich, T.A. & Guffanti, A.A. (1989).
Alkalophilic bacteria.
Annual Review of Microbiology **43**, 435-463.

Kuritzkes, D.R., Zhang, X.-Y. & Lin, E.C.C. (1984).
Use of ϕ (glp-lac) in studies of respiratory regulation of the *Escherichia coli* anaerobic sn-glycerol-3-phosphate dehydrogenase genes (glpAB).
Journal of Bacteriology **157**, 591-598.

Kwan, H.B. & Barrett, E.L. (1983).
Roles for menaquinone and the two trimethylamine oxide (TMAO) reductases. *In* TMAO reductases in TMAO respiration in *Salmonella typhimurium*: Mud(Apr-*lac*) insertion mutations in *mes* and *tor*.
Journal of Bacteriology **155**, 1147-1155.

Kwan, H.S. & Barrett, E.L. (1985).
Bacterial reduction of trimethylamine oxide.
Annual Review of Microbiology **39**, 131-149.

Laemmli, U.K. (1970).
Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature **227**, 680-685.

Lambden, P.R. & Guest, J.R. (1976).
A novel method for isolating chlorate-resustant mutants of *E. coli* K12 by anaerobic selection on a lactate plus fumarate medium.
Journal of General Microbiology **93**, 173-176.

Lambden, P.R. & Guest, J.R. (1976).
Mutants of *E. coli* unable to use fumarate as an anareobic electron acceptor.
Journal of General Microbiology **97**, 145-160.

Langley, K.E., Villarejo, M.R., Fowler, A.U., Zamenhol, P.J. & Zabih, I. (1975)
Molecular basis of β-galactosidase a-complementation. Proceedings of the National Academy of Science USA **72**, 1254-1257.

Laszlow, D.J., Fandrich, B.L., Suraran, A., Chance, B. & Taylor, B.L. (1984).
Cytochrome o as a terminal oxidase and receptor for aerotaxis in *Salmonella typhimurium*.
Journal of Bacteriology **159**, 663-667.

Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G. & Maniatis, T. (1978).
The isolation and characterisation of linked and β-globin genes from a cloned library of human DNA.
Cell **15**, 1157-1174.

Leder, P., Tiemer, D. & Enquist, L. (1977).
EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the λgt WES system.
Science **196**, 175-177.

Lee, C.C.T. & Merkel, J.R. (1981).
Selective release and purification of two periplasmic *Alteromonas* B-207 amino peptidases.
Biochimica et Biophysica Acta **661**, 39-44.

Leemans, R., Remaut, E. and Fiers, W. (1987).
A broad-host-range expression vector based on the PL promoter of coliphage λ: Regulated synthesis of human interleukin 2 in *Erwinia* and *Serratia* species.
Journal of Bacteriology **169**, 1899-1904.

Leemans, J., Langenakens, J., deGreve, H., Deblore, R., Van Montagu, M. & Schell, J. (1982)
Broad host-range cloning vectors derived from the W-plasmid Sa.
Gene **19**, 361-364.

Lemire, B.D., Robinson, J.J. & Weiner, J.H. (1982).
Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase.
Journal of Bacteriology **152**, 1126-1131.

Leong, S.A., Ditta, G.S. & Helsinki, D.R. (1982).
Heme biosynthesis in *Rhizobium*.
Journal of Biological Chemistry **257**, 8724-8730.

Lester, R.L. & DeMoss, J.A. (1971).
Effects of molybdate and selenite on formate and nitrate metabolism in *Escherichia coli*.
Journal of Bacteriology **105**, 1006-1014.

Lewin, B. (1990).
Genes (4th Edition). Oxford University Press.

Lin, E.C.C. & Kuritzkes, D.R. (1987).
Pathways for anaerobic electron transport, p201-221.
In F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, H. Schaechler & H.E. Umborger (ed), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, DC.

Loheimer, E., Hagen, D.S., Dickie, P. & Weiner, J.H. (1981).
Cloning and expression of the fumarate reductase gene of *Escherichia coli*.
Canadian Journal of Biochemistry **59**, 158-164.

Lowry, O.H., Rosenburgh, N.J., Fahr, A.J. & Randell, R.J. (1951).
Protein measurements with the Folin-Phenol reagent.
Journal of Biological Chemistry **193**, 265-275.

MacGregor, C.H. (1975). Synthesis of nitrate reductase components in chlorate-resistant mutants of *Escherichia coli*.
Journal of Bacteriology **121**, 1117-1121.

MacGregor, C.H. & Christopher, A.R. (1978).
Assymetric distribution of nitrate reductase subunits in the cytoplasmic membrane of *Escherichia coli*: evidence derived from surface labelling studies with transglutaminase.
Archives of the Biochemistry and Biophysics **185**, 204-213.

MacGregor, C.H. & Schnaitman, C.A. (1973).
Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*.
Journal of Bacteriology **114**, 1164-1176.

MacInnes, J.I., Kim, J.E., Lian, C.-J. & Soltes, G.A. (1990).
Actinobacillus pleuropneumoniae hylX gene homology with the *fnr* gene of *Escherichia coli*.
Journal of Bacteriology **172**, 4587-4592.

Mandel, M. & Higa, A. (1970).
Calcium-dependent bacteriophage DNA infection.
Journal of Molecular Biology **53**, 159-162.

Mandelstam, J., McQuillen, K. & Dawes, I. (1982).
Biochemistry of Bacterial Growth. Blackwell Scientific Publications.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1989).
Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press.

McEwan, A.G., Donohue, T.S. & Kaplan, S. (1989).
Synthesis of *Rhodobacter sphaeroides* cytochrome c_2 in *Escherichia coli*.
FEMS Microbiology Letters **59**, 253-258.

McEwan, A.G., Wetzstein, H.G., Meyer, O., Jackson, J.B. & Ferguson, S.J. (1987).
The periplasmic nitrate reductase of *Rhodobacter capsulatis*: purification, characterisation and distinction from a single reductase for trimethylamine-N-oxide, dimethylsulphoxide and chlorate.
Archives of Microbiology **147**, 340-345.

McIntire, W. & Singer, T.P. (1982).
Resolution of *p*-cresol methylhydroxylase into catalytically active subunits and reconstitution of the flavocytochrome.
FEBS Letters **143**, 316-318.

McIntire, W., Edmonson, D.E., Hopper, D.J. & Singer, T.P. (1981).
8α-(o-tryosyl) flavin adenine dinucleotide, the prosthetic group of bacterial p-cresol methyl hydroxylase.
Biochemistry **20**, 3068-3075.

McIntire, W., Singer, T.P., Smith, A.J. & Mathews, F.S. (1986).
Amino acid and sequence analysis of the cytochrome and flavoprotein subunits of *p*-cresol methyl hydroxylase.
Biochemistry **25**, 5975-5981.

McLendon, G., Hake, R., Zhang, Q.P. & Simmons, J. (1991).
Coupling of interfacial motion in protein complexes to intra complex electron transfer.
Journal of Inorganic Biochemistry **43**, 89.

Merkel, J. R., Lee, C.C.T. & Freund, T.S. (1980).
A dimeric extracellular heat stable amino peptidase produced by a marine pseudomonad.
Biochimica et Biophysica Acta **661**, 32-38.

Messing, J. (1983).
New M13 vectors for cloning. *In* Methods in Enzymology (R. Wu, L. Grossman & K. Moldave eds), vol 101, pp20-78. Academic Press, Orlando.

Meyer, T.E. & Bartsch, R.G. (1976).
The reaction of flavocytochromes *c* of the phototrophic sulphur bacteria with thiosulfate, cyanide and mercaptans. *In* Flavins and Flavoproteins (Singer, T.P. ed), Elsevier, Amsterdam. 312.

Meyer, T.E., Bartsch, R.G., Cusanovich, M.A. and Mathewson, J.H., (1968).
The cytochromes of *Chlorobium thiosulfatophilum*.
Biochimica et Biophysica Acta **153**, 854.

Meyer, T.E., Vorkink, W.P., Tollin, G. & Cusanovich, M.A. (1985).
Chromatium flavocytochrome *c*: Kinetics of reduction of the heme subunit, and the flavocytochrome *c*-mitochondrial cytochrome *c* complex.
Archives in Biochemistry and Biophysics **236**, 52-58.

Mierendorf, R.C., Percy, C. & Young, R.A. (1987).
Gene isolation by screening λgt11 libraries with antibodies.
Methods in Enzymology **152**, 458.

Miles, C.S., Reid, G.A. & Chapman, S.K. (1991).
Mutagenesis of an iron ligand, His-43, in flavocytochrome b_2: effects on enzymatic activity.
Journal of Inorganic Biochemistry **43**, 313.

Miller J.B. & Amy, N.K. (1983).
Molybdenum cofactor in chlorate-resistant and nitrate reductase-deficient insertion mutants of *Escherichia coli*.
Journal of Bacteriology **155**, 793-801.

Miller, J.F., Dower, W.J. & Tompkins, L.J. (1988).
High voltage electroporation of bacteria. Genetic transformation of *Campylobacter jejuni* with plasmid DNA.
Proceedings of the National Academy of Science USA **85**, 856.

Mitchell, P. (1979).
Keilin's respiratory chain concept and its chemiosmotic consequences.
Science **206**, 1148-1159.

Moodie, A.D. & Ingledew, W.J. (1990).
Microbial anaerobic respiration.
Advances in Microbial Physiology **31**, 225-269.

Morningstar, J.E., Johnson, M.K., Ceccini, G., Ackrell, B.A.C. & Kearney, E.B. (1985).
The high potential iron-sulfur center in *Escherichia coli* fumarate reductase is a three-iron cluster.
Journal of Biological Chemistry 260, 13631-13638.

Morris, C.J. (1987).
The cytochromes of *S. putrefaciens*.
PhD thesis, Edinburgh University.

Morris, C.J., Gibson, D.M. & Ward, F.B. (1990).
Influence of respiratory substrate on the cytochrome content of *Shewanella putrefaciens*.
FEMS Microbiology Letters **69**, 259-262.

Moser, C.C., Warncke, K., Keoke, J.M. & Dutton, P.L. (1991).
The nature of protein mediated electron transfer.
Journal of Inorganic Biochemistry **43**, 91.

Mount, D.W. (1980).
The genetics of protein degradation in bacteria.
Annual Review of Genetics **14**, 279-319.

Muller-Hill, B., Crapo, L. & Gilbert, W. (1968).
Mutants that make more *lac* repressor.
Proceedings of the National Academy of Science USA **59**, 1259-1264.

Muratsubaki, H. & Katsume, T. (1982).
Purification and properties of fumarate reductase from Baker's yeast.
Agricultural and Biological Chemistry **46**, 2909-2917.

Muratsubaki, H. & Katsume, T. (1985).
Characterisation of fumarate reductase from Baker's yeast: essential sulfhydryl group for binding of FAD.
Journal of Biochemistry **97**, 1201-1209.

Murray, N. (1983).
Phage lambda and molecular cloning.
In *Lambda II*, pp395-432. Eds R. Hendrix, J. Roberts, F. Stahl & R. Weisberg. Cold Spring Harbor: New York.

Nasser, Z. M. (1983).
Physiological studies of trimethylamine oxide reduction of *Alteromonas putrefaciens*.
PhD thesis, University of Edinburgh, Scotland.

Nathan, J. & Hogness, D.S. (1983).
Isolation, sequence analysis and intron-exon arrangement of the gene encoding bovine rhodospin.
Cell **34**, 807-814.

Newman, M.B. & Cole, J.A. (1978).
The chromosomal location and pleiotrophic effects of mutations of the *nirA*$^+$ gene of *Escherichia coli* K12: The essential role of *nirA*$^+$ in nitrate reduction and in other anaerobic redox reactions.
Journal of General Microbiology **106**, 1-12.

Ni Briain, N., Dorman, C.J. & Higgins, C.F. (1989).
An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression.
Molecular Microbiology **3**, 933-942.

Nichols, B.P. & Yanofsky, C. (1979).
Nucleotide sequences of trpA of *S. typhimurium* and *E. coli*. An evolutionary comparison.
Proceedings of the National Academy of Science USA **70**, 5244-5248.

Nilsson, B., Uhlen, M., Josephson, S., Galenbeck, S. & Philipson, L. (1983).
An improved positive plasmid selection vector constructed by oligonucleotide mediated mutagenesis.
Nucleic Acids Research **11**, 8019-8030.

Nishikimi, M., Suzuki, H., Ohta, S., Sakurai, T., Shimomura, Y., Tanaka, M., Kagawa, Y. & Ozawa, T. (1987).
Isolation of a cDNA clone for human cytochrome c_1 from a λgt11 expression library.
Biochemical and Biophysical Research Communications **145**, 34-39.

Nishimura, Y., Tan, I.K.P., Ohgami, V., Kahgami, N. & Hamimura, T. (1983).
Induction of membrane-bound L-lactate dehydrogenase in *E. coli* under conditions of nitrate respiration, fumarate reduction and trimethylamine-N-oxide reduction.
FEMS Letters **17**, 283-286.

Norrander, J., Kempe, T. & Messing, J. (1983).
Construction of improved M13 vectors during oligo-deoxynucleotide-directed mutagenesis.
Gene **26**, 101-106.

Odom, J.M. & Peck, H.D., Jr (1984).
Hydrogenase, electron transfer protein and energy coupling in the sulfate-reducing bacterium *Desulfovibrio*.
Annual Review of Microbiology **38**, 551-592.

Old, R.W. & Primrose, S.B. (1985).
Principles of gene manipulation.
Blackwell Scientific Publications.

Oltmann, L.F., Reijnders, W.N.M. & Stouthamer, A. H. (1976).
Characterisation of purified nitrate reductase A and chlorate reductase C from *Proteus mirabilis*.
Archives of Microbiology **111**, 25-35.

Page, M.D. & Ferguson, S.J. (1989).
A periplasmic c-type cytochrome can be translocated to the periplasm as an apo form; the biosynthesis of nitrite reductase of *Paracoccus denitrificans*.
Molecular Biology **3**, 653-661.

Page, M.D. & Ferguson, S.J. (1990).
Apo-forms of cytochrome c_{550} and cytochrome cd_1 are translocated to the periplasm of *Paracoccus denitrificans* in the absence of haem incorporation caused by either mutation or inhibition of haem synthesis.
Molecular Biology **4**, 1181-1192.

Perry, D.N. (1989).
Isolation of fumarate reductase mutants of *Shewanella putrefaciens*.
Honours thesis, Edinburgh University Microbiology Department.

Peterson, G.L. (1977)
A simplification of the protein assay method of Lowry *et al* which is more generally applicable.
Analytical Biochemistry **83**, 346-356.

Pettigrew, G.W. & Moore, G.R. (1987).
Cytochromes c. Biological Aspects Springer Verlag.

Phillips, M.K., Hederstedt, L., Hasnain, S., Rutberg, L. & Guest, J.R. (1987).
Nucleotide sequence encoding the flavoprotein and iron-sulphur protein subunits of the *Bacillus subtilis* PY79 succinate dehydrogenase complex.
Journal of Bacteriology **169, 864-873.**

Pouwels, P.H., Enger-Valk, B.E. and Brammar, W.J. (1985).
Cloning vectors, a laboratory manual. Elsevier.

Powell, I.B., Achen, M.G., Hillier, A.J. & Davidson, B.E. (1988).
A simple and rapid method for genetic transformation of lactic streptococci by electroporation.
Applied and Environmental Microbiology, **54**, 655-660.

Pugsley, E.P. & Schwartz, M. (1985).
Export and secretion of proteins by bacteria.
FEMS Microbiology Reviews **32**, 3-38.

Reeve, C.D., Moreman, A.D. & Hopper, D.J. (1988).
Alkylophenol hydroxylases in the oxidation of *p*-cresol, 4-ethylphenol and 4-n-propylphenol by *Pseudomonas putida* JD1.
FEMS Microbiology Letters **52**, 251.

Remaut, E., Tsao, H. & Fiers, W. (1983).
Improved plasmid vectors with a thermoinducable expression and a temperature-regulated runaway replication.
Gene **22**, 103-113.

Rice, D.W., Schultz, G.E. & Guest, J.R. (1984).
Structural relationship between glutathione reductase and lipoamide dehydrogenase.
Journal of Molecular Biology **174**, 483-496.

Ringo, E., Stenberg, E. & Strom, A.R. (1984).
Amino acid and lactate catabolism in trimethylamine oxide respiration of Alteromonas putrefaciens NCMB1735.
Applied and Environmental Microbiology **47**, 1084-1089.

Riviere, C., Giordono, G., Pommier, J. & Azoulay, E.J. (1975).
Membrane reconstitution in *chlr-r* mutants of *Escherichia coli* K12. VIII. Purification and properties of the F_A factor, the product of the *chlB* gene.
Biochimica et Biophysica Acta **389**, 219-235.

Robinson, J.J. & Weiner, J.H. (1982).
Molecular properties of fumarate reductase isolated from the cytoplasmic membrane of *Escherichia coli*.
Canadian Journal of Biochemistry **60**, 811-816.

Rokeach, L.A., Haselby, J.A. & Hoch, S.O. (1988).
Molecular cloning of a cDNA encoding the human SM-D autoantigen.
Proceedings of the National Academy of Science USA **85**, 4832-4836.

Ruiz-Herera, J. & DeMoss, J.A. (1969).
Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome b_1 components in nitrate reduction.
Journal of Bacteriology **99**, 720-729.

Ruiz-Herera, J., Showe, M.K. & DeMoss, J.A. (1969).
Nitrate reductase complex of *Escherichia coli* K-12: isolation and characterisation of mutants unable to reduce nitrate.
Journal of Bacteriology **97**, 1291-1297.

Ruther, U. & Muller-Hill, B. (1983).
Easy identification of cDNA clones.
The EMBO Journal **2**, 1791-1794.

Sagai, M. & Ishimoto, M. (1973).
An enzyme reducing adenosine N-oxide in *Escherichia coli*, amine N-oxide reductase.
Journal of Biochemistry **73**, 843-859.

Sakaguchi, M. & Kawai, A. (1975).
Inhibition of trimethylamine N-oxide reductase formation by nitrate in *Escherichia coli*.
Bulletin of the Japanese Society of Scientific Fisheries **41**, 437-442.

Sakaguchi, M. & Kawai, A. (1977).
Electron donors and carriers for the reduction of trimethylamine N-oxide in *Escherichia coli*.
Bulletin of the Japanese Society of Scientific Fisheries **43**, 437-442.

Sakaguchi, M. & Kawai, A. (1979).
Presence of *b*- and *c*-type cytochromes in the membrane of *Escherichia coli* induced by trimethylamine N-oxide.
Bulletin of the Japanese Society of Scientific Fisheries **44**, 999-1002.

Sanchez-Crispin, J.A., Dobourdieu, A.M. & Chippaux, H. (1979).
Localisation and characterisation of cytochromes from membrane vesicles of *Escherichia coli* K-12 grown anaerobically with nitrate.
Biochimica et Biophysica Acta **547**, 198-210.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977).
DNA sequencing with chain terminating inhibitors.
Proceedings of the National Academy of Science USA **74**, 5463-5467.

Schulz, G.E. & Schirmer, R.H. (1979).
Principles of protein structure, pp128-129. Springer Verlag, New York.

Scolnik, A.P. & Marrs, B.L. (1987).
Genetic research with photosynthetic bacteria.
Annual Review of Microbiology **41**, 703-726.

Scopes, R. (1982).
Protein purification principles and practice. Springer-Verlag New York Incorporated.

Sela, M., Schechter, B., Schechter, I. & Barek, F. (1967).
In Cold Spring Harbor Symposium Quantitative Biology **32**, 537-545.

Shaw, D.J. & Guest, J.R. (1981).
Molecular cloning of the *fnr* gene of *Escherichia coli* K12.
Molecular and General Genetics **181**, 95-100.

Shaw, D.J. & Guest, J.R. (1982a).
Amplification and product identification of the *fnr* gene of *Escherichia coli*.
Journal of General Microbiology **128**, 2221-2228.

Shaw, D.J. & Guest, J.R. (1982b).
Nucleotide sequence of the *fnr* gene and primary structure of the Fnr protein of *Escherichia coli*.
Nucleic Acids Research **10**, 6119-6130.

Shaw, D.J., Rice, D.W. & Guest, J.R. (1983).
Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*.
Journal of Molecular Biology **166**, 241-247.

Sherratt, D. J. (1982).
The maintenance and propogation of plasmid genes in bacterial populations.
The Journal of General Microbiology **128**, 655-661.

Shimokawa, O. & Ishimoto, M. (1979).
Purification and some properties of inducible tertiary amine N-oxide reductase from *E. coli*.
Journal of Biochemistry (Tokyo) **86**, 1709-1777.

Shine, J. & Dalgarno, L. (1975).
Determinant of cistron specificity in bacterial ribosomes.
Nature, London **299**, 265-268.

Simpkin, D. & Ingledew, W.J. (1985).
The membrane-bouns fumarate reductase of *Escherichia coli*: an electron-paramagnetic-resonance study.
Biochemical Society Transactions **13**, 603-607.

Singh, A.P. & Bragg, P.D. (1975).
Reduced nicotinamide adenine dinucleotide dependent reduction of fumarate coupled to membrane energization in a cytochrome deficient mutant of *Escherichia coli* K12.
Biochimica et Biophysica Acta **396**, 229-241.

Smith, M.W. & Neidhart, F.C. (1983).
Proteins induced by anaerobiosis in *Escherichia coli*.
Journal of Bacteriology **154**, 336-343.

Sodergren, E.J. & DeMoss, J.A. (1988).
narI region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes.
Journal of Bacteriology **170**, 1721-1729.

Southern, E.M. (1975).
Detection of specific sequences among DNA fragments separated by gel electrophoresis.
Journal of Molecular Biology **98**, 503-517.

Spence, J.T. (1980).
Model reactions of molybdenum complexes.
In Molybdenum and Molybdenum-containing Enzymes (ed Coughlan, H.). Permagon Press Ltd, Oxford, pp99-118.

Spencer, M. E. & Guest, J.R. (1973).
Isolation and properties of fumarate reductase mutants of *E.coli*.
Journal of Bacteriology **114**, 563-570.

Spencer, M.E. & Guest, J.R. (1985).
Transcription analysis of the *sucAB, aceEF* and *lpd* genes of *Escherichia coli*.
Molecular and General Genetics **200**, 145-154.

Spencer, M.E. & Guest, J.R. (1987).
Regulation of citric acid cycle genes in facultative bacteria.
Microbiological Science **4**, 164-168.

Sperl, G. T. & DeMoss, J. A. (1975).
chlD gene function in molybdate activation of nitrate reductase.
Journal of Bacteriology **122**, 1230-1238.

Spiro, S. & Guest, J.R. (1987).
Regulation and over-expression of the *fnr* gene of *Escherichia coli*.
Journal of General Microbiology **133**, 3279-3288.

Spiro, S. & Guest, J.R. (1988).
Inactivation of the Fnr protein of *Escherichia coli* by targeted mutagenesis in the N-terminal region.
Molecular Microbiology **2**, 701-707.

Spiro, S. & Guest, J.R. (1990).
FNR and its role in oxygen-regulated gene expression in *Escherichia coli*.
FEMS Microbiology Reviews **75**, 399-428.

Spiro, S. & Guest, J.R. (1991).
Adaptive responses to oxygen limitation in *Escherichia coli*.
TIBS **16**, 310-314.

Spizizen, J., Reilly, B.E. & Evans, A.H. (1966).
Microbial transformation and transfection.
Annual Review of Microbiology **20**, 371-400.

Stanley, K.K. & Luzio, J.P. (1984).
Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins.
The EMBO Journal **3**, 1429 - 1434.

Steinbuch, A & Audran (1969).
The isolation of IgG from mammalian sera with the aid of caprylic acid.
Archives of Biochemistry and Biophysics **134**, 279-284.

Steinmetz, M.A. & Fischer, U. (1981).
Cytochromes of the non-thiosulfate-utilizing green sulfur bacterium *Chlorobium limicola*.
Archives in Microbiology **130**, 31.

Stellwagen, E., Rysavy, R. & Babul, G. (1972).
The conformation of horse heart cytochrome c.
Journal of Biological Chemistry **247**, 8074-8077.

Stenberg, E., Ringo, E. & Strom, A.R. (1984).
Trimethylamine N-oxide respiration of *A. putrefaciens* NCMB1735: Na stimulated anaerobic transport in cells and membrane vesicles.
Applied and Environmental Microbiology **47**, 1090-1095.

Stewart, G.S.A.B., Lubinsky-Mink, S., Jackson, C.G., Cassel, A. & Kuhn, J. (1986a).
pHG165: A pBR322 copy number derivative of pUC8 for cloning and expression.
Plasmid **15**, 172-181.

Stewart, G.S.A.B., Lubinsky-Mink, S. & Kuhn, J. (1986b).
pHG276: A multiple cloning site pBR322 copy number vector expressing a functional lac peptide from the bacteriophage lambda PR promoter.
Plasmid **15**, 182-190.

Stewart, V. (1982).
Requirement of fnr and narL functions for nitrate reductase expression in *Escherichia coli* K-12.
Journal of Bacteriology **151**, 1320-1325.

Stewart, V. (1988).
Nitrate respiration in relation to facultative metabolism in Enterobacteria.
Microbiological Reviews **52**, 190-232.

Stewart, V. & MacGregor, C.H. (1982).
Nitrate reductase in *Escherichia coli* K-12: Involvement of chlC, chlE, and chlG loci.
Journal of Bacteriology **151**, 788-799.

Stewart, V. & Parales Jr, J. (1988).
Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12.
Journal of Bacteriology **170**, 1589-1597.

Stewart, V. & Parales Jr, J. & Merkel, S.M. (1989).
Structure of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12.
Journal of Bacteriology **171**, 2229-2234.

Stock, J.B., Ninfa, A.J. & Stock, A.M. (1989).
Protein phosphorylation and regulation of adaptive responses in bacteria.
Microbiological Reviews **53**, 450-490.

Strauch, K.L., Botti Lenk, J., Gamble, B.L. & Miller, C.G. (1985).
Oxygen Regulation in *Salmonella typhimurium*.
Journal of Bacteriology **161**, 673-680.

Strom, A. R. & Larsen, H. (1979).
Anaerobic fish spoilage bacteria. I. Biochemical changes in herring extracts.
Journal of Applied Bacteriology **46**, 531-543.

Strom, A. R., Olafsen, J. A. & Larsen, H. (1979).
Trimethylamine oxide: a terminal electron acceptor in anaerobic respiration bacteria.
The Journal of General Microbiology **112**, 315-320.

Stupp, Y., Paul, W.E. & Benacerraf, B. (1971).
Structural control of immunogenicity. II. Antibody synthesis and cellular immunity in response to immunisation with mono-ϵ-oligo-L-lysines.
Immunology **21**, 583-594.

Styrudd, O.B. & Strom, A.R. (1984).
Dimethylsulphoxide and trimethylamine oxide respiration of *Proteus vulgaris*. Evidence for a common terminal reductase system.
Archives of Microbiology **140**, 74-78.

Suzuki, M. & Szalay, A.A. (1979)
Bacterial transformation using temperature sensitive mutants deficient in peptidoglycan synthesis.
Methods in Enzymology **68**, 334-342.

Takagi, M. & Ishimoto, M. (1983).
E. coli mutants defective in trimethylamine N-oxide reductase.
FEMS Microbiology Letters **17**, 247-250.

Takagi, M., Tsuchiya, T. & Ishimoto, M. (1981).
Proton translocation coupled to TMAO reduction in anaerobically grown *Escherichia coli*.
Journal of Bacteriology **148**, 762-768.

Taxeira de Mattos, M.J., Streekstra, H. & Tempest, D. W. (1984).
Metabolic uncoupling of substrate-level phosphorylation in anaerobic glucose-limited chemostat cultures of *Klebsiella aerogenes* NCTC 418.
Archives of Microbiology **139**, 260-264.

Taylor, S.S. (1977).
Amino acid sequence of dogfish muscle lactate dehydrogenase.
Journal of Biological Chemistry **252**, 1799-1806.

Thomas, A.D., Doelle, H.W., Westward, A.W. & Gordon, G.L. (1972).
Effect of oxygen on several enzymes involved in the aerobic and anaerobic utilisation of glucose in *Escherichia coli*.
Journal of Bacteriology **112**, 1098-1105.

Thomas, M., Cameron, J.R. & Davis, R.W. (1974).
Viable molecular hybrids of bacteriophage lambda and eukaryotic DNA.
Proceedings of the National Academy of Science USA **71**, 4579.

Thomas, P.E., Ryan, D. & Levin, W. (1976).
An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels.
Analytical Biochemistry **75**, 168-176.

Thorneley, R.N.F. (1974).
A convenient electrochemical preparation of reduced methyl viologen and a kinetic study of the reaction with oxygen using an anaerobic stopped-flow apparatus.
Biochimica et Biophysica Acta **333**, 487-496.

Todd, P.E.E., East, I.J. & Laech, S.J. (1982).
The immunogenicity and antigenicity of proteins.
Trends in Biochemical Sciences, 212-216.

Tollin, G., Meyer, T.E. & Gusanovich, M.A. (1986).
Elucidation of the factors which determine reaction-rate constants and biological specificity for electron-transfer proteins.
Biochimica et Biophysica Acta **853**, 29-41.

Twigg, A.J. & Sherratt, D. (1980).
Trans-complementable copy-number mutants of plasmid colE1.
Nature, London **283**, 216-218.

Ugalde, R.A., Imperial, J., Shah, U.K. & Brill, W.J. (1985).
Biosynthesis of the iron-molybdenum cofactor and the molybdenum cofactor in *Klebsiella pneumonia*: effect of sulphur source.
Journal of Bacteriology **164**, 1081-1087.

Unden, G. & Cole, S.T. (1983).
Studies on the relatedness of bacterial fumarate reductases.
FEMS Microbiology Letters **20**, 181-185.

Unden, G. & Duchene, A. (1987).
On the role of cyclic AMP and the Fnr protein in *Escherichia coli* growing anaerobically.
Archives of Microbiology **147**, 195-200.

Unden, G. & Kroger, A. (1980).
An essential sulfhydryl group at the substrate site of the fumarate reductase of *Vibrio succinogenes*.
FEBS Letters **117**, 323-326.

Unden, G., Hackenberg, H. & Kroger, A. (1980).
Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of *Vibrio succinogenes*.
Biochimica et Biophysica Acta **591**, 275-288.

Unemoto, T., Hayashi, M., Miyaki, K. & Itagaki, M. (1965).
Intracellular localisation and properties of trimethylamine-N-oxide reductase in *V. parahaemolyticus*.
Biochimica et Biophysica Acta **110**, 319-328.

Van Wielink, J.E., Reijnders, W.N.M., Oltmann, L.F., Leuweink, F.J. & Stouthamer, A.H. (1983).
The membrane bound *b* and *c*-type cytochromes of *Proteus mirabilis* grown under different conditions. Characterisation by means of coupled spectrum deconvolution and potentiometric analysis.
Archives of Microbiology **134**, 118-122.

Van't Riet, J. & Planta, R.S. (1975).
Purification, structure and properties of the respiratory nitrate reductase of *Klebsiella aerogenes*.
Biochimica et Biophysica Acta **379**, 81-94.

Van't Riet, J., Stouthamer, A.H. & Planta, R.J. (1968).
Regulation of nitrate assimilation and nitrate respiration in *Aerobacter aerogenes*.
Journal of Bacteriology **96**, 1455-1464.

Viera, J. & Messing, J. (1982).
The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with universal primers.
Gene **19**, 259-268.

Vik, S. B. & Hatefi, Y. (1981).
Possible occurrence and role of an essential histidyl residue in succinate dehydrogenase.
Proceedings of the National Academy of Science USA **78**, 6749-6753.

Voordouw, G. & Brenner, S. (1986).
Cloning and sequencing of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough).
European Journal of Biochemistry **159**, 347-351.

Walker, W.H., Kenney, W.C., Edmondson, D.E., Singer, T.P., Cronin, J.R. & Hendriks, R. (1974).
The covalently bound flavin of *Chromatium* cytochrome c-552. 1. Evidence for cysteine thiohemiacetal at the 8α position.
European Journal of Biochemistry **48**, 439-448.

Wallace, B.J. & Young, I.G. (1977a).
Aerobic respiration in mutants of *Escherichia coli* accumulating quinone analogs of ubiquinone.
Biochimica et Biophysica Acta **461**, 75-83.

Wallace, B.J. & Young, I.G. (1977b).
Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with a *ubiA menA* double quinone mutant.
Biochimica et Biophysica Acta **461**, 84-100.

Warren, M.J. & Scott, A.I. (1990).
Tetrapyrate assembly and modification into the ligands of biologically functional cofactors.
TIBS **15**, 486-491.

Way, J.C, Davis, M.A., Morisato, D., Roberts, D.E. & Kledener, N. (1984).
New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition.
Gene **32**, 369-379.

Weiner, J.H. & Dickie, P. (1979). Fumarate reductase of *Escherichia coli* - elucidation of the covalent-flavin component.
Journal of Biological Chemistry **254**, 8590-8593.

Weiner, J.H., MacIsaac, D.P., Bishop, R.E. & Bilous, P.T. (1988).
Purification and properties of *Escherichia coli* dimethyl sulfoxide, an iron-sulfur molybdoenzyme with broad substrate specificity.
Journal of Bacteriology **170**, 1505-1510.

Wierenga, R.K., Terpstra, P. & Hol, W.J.G. (1986).
Prediction of the occurrence of the ADP-binding bab-fold in proteins, using an amino acid sequence fingerprint.
Journal of Molecular Biology **187**, 101-107.

Williams, C.H., Jr, Thrope, C. & Arscott, L.D. (1978).
Developments in Biochemistry **1** (Singer, T. P. & Ondoza, R.N. eds). Elsevier/North Holland, New York.

Wood, D., Darlison, M.G., Wilde, R.J. & Guest, J.R. (1984).
Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*.
Biochemical Journal **222**, 519-534.

Wood, W.A. (1961).
Fermentation of carbohydrates and related compounds, p59-149.
In I.C. Gunsalus & R. Y. Stanier (ed), The Bacteria. A treatise on structure and function, vol 2, Academic Press Inc, New York.

Yamamoto, I. & Ishimoto, M. (1977).
Anaerobic growth of *E. coli* on formate by reduction of nitrate, fumarate and trimethylamine N-oxide.
Zeitschrift fur Allegmeine Mikrobiogie **17**, 235-242.

Yamanaka, T. (1976).
The subunits of *Chlorobium* flavocytochrome *c*.
Journal of Biochemistry **79**, 655-660.

Yamanaka, T. & Kusai, A. (1976).
The function and some molecular features of cytochrome *c*-553 derived from *Chlorobium thiosulfatophilum*.
In Flavins and Flavoproteins, Singer, T.P. (ed), Elsevier, Amsterdam. 292.

Yamanaka, T., Fukumori, Y. & Okun uki, K. (1979).
Preparation of subunit of flavocytochromes *c* derived from *Chlorobium limicola f* and *Chromatium vinosum*.
Analytical Biochemistry **95**, 209.

Yanofsky, C. & Horn, V. (1981).
Rifampicin resistance mutations that alter the efficiency of transcription termination at the tryptophan operon attenuator.
Journal of Bacteriology **145**, 1334-1341.

Young, R.A. & Davis, R.W. (1983).
Efficient isolation of genes by using antibody probes.
Proceedings of the National Academy of Science USA **80**, 1194-1198.

Young, R.A. & Davis, R.W. (1984).
Yeast RNA polymerase II genes: isolation with antibody probes.
Science **222**, 778-782.

Zabeau, M. & Stanley, K.K. (1982).
Enhanced expression of Cro-β-galactosidase fusion protein under the control of the P_R promoter of bacteriophage λ.
EMBO Journal **1**, 1217-1224.

Zimmerman, S.B. (1982).
Bacterial transformation using temperature-sensitive mutants deficient in peptidoglycan synthesis
Biochimica et Biophysica Acta **694**, 227-277.