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Studies on the filamentous gliding bacteria *Vitreoscilla stercoraria*

Biggs, Laura Frances

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University of Plymouth

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**Studies on the filamentous gliding bacteria
*Vitreoscilla stercoraria***

By

Laura Frances Biggs

A thesis submitted to the University of Plymouth in partial fulfilment for
the degree of

Doctor of Philosophy

School of Biological Sciences
Faculty of Science

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Abstract

Strains of *Vitreoscilla stercoraria* were isolated from the environment and characterised. Cell width, motility and requirement of each strain for sodium chloride were investigated. Two strains were selected for further study and the effect of monensin and FCCP on growth of the strains was investigated. One strain of *Vitreoscilla* (LB13) was chosen for further study, cells from strain LB13 were found to be $1.38 \mu\text{m} \pm 0.041$ (± 1 SEM, $n=10$) wide, were motile by gliding and had an optimum requirement for sodium chloride for growth of 43 mM. The organism was grown in batch culture and respiratory membranes were isolated. Cytochrome *bo* was extracted from the respiratory membranes and further purification was achieved using column chromatography. A yield of 6.71 % was achieved with a purification factor of 18.5. The light sensitivity of *Vitreoscilla stercoraria* was investigated. Two strains of *Vitreoscilla* (LB13 and C1) were shown to be highly sensitive to UV-A (320-400 nm) with an LD_{50} of less than 20 kJm^{-2} . Superoxide dismutase and catalase were shown to provide protection from the effect of UV-A during exposure, either separately or together, indicating an involvement of reactive oxygen species. A photo-insensitive strain (LB13A) was isolated during an exposure experiment and originated from a culture of LB13. The possible sodium pumping activity of cytochrome *bo* from two strains of *Vitreoscilla* (LB13 and C1) was investigated. The V_{max} of decylubiquinol oxidation by respiratory membranes from LB13 and C1 were calculated and found to be $0.96 \text{ nmol s}^{-1} \text{ mg}^{-1}$ and $13.33 \text{ nmol s}^{-1} \text{ mg}^{-1}$ respectively. The K_m of decylubiquinol oxidation by LB13 membranes was found to be $9.8 \mu\text{M}$. Quinol oxidation activity was tested for dependence on sodium chloride in both respiratory membranes and in the purified enzyme. No stimulation of activity was shown with either strain

using decylubiquinol, duroquinol or menadiol as substrates. Given the lack of sodium sensitivity it is unlikely that the enzyme pumps sodium.

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Author's declaration

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Signed

Date

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Chapter one

Introduction

1.1 Vitreoscilla

1.1.1 Classification

The family *Vitreoscillaceae* was first described in 1949, and contained the genus *Vitreoscilla* along with other colourless, gliding bacteria (Pringsheim, 1949). In 1951 Pringsheim characterised the genus *Vitreoscilla* in more detail. He proposed a new family, the *Vitreoscillaceae*, characterised by their colourless, gliding trichomes. Pringsheim proposed two genera, *Vitreoscilla* and *Microscilla* and seven species were described (three *Vitreoscilla*, two *Microscilla* and two others) (Pringsheim, 1951). Like some other gliding bacteria, for example *Myxobacteria*, *Vitreoscilla* produce chains of cells called trichomes. In *Vitreoscilla filiformis* and *Vitreoscilla beggiatoides* trichomes the cells are in close contact over a large area; this differs from chains found in other bacteria such as *Bacillus* which maintain contact in only a small area. *Vitreoscilla stercoraria* trichomes resemble other bacterial chains in that contact is made over a small area. Electron micrographs of *Vitreoscilla stercoraria* show separate cells joined together by electron dense material (Strohl, Schmidt et al., 1986). *Vitreoscilla* trichomes are more pronounced than those produced by *Myxobacteria*. Another definitive characteristic of *Myxobacteria* is the formation of microcysts. Microcysts form in response to harsh or challenging environmental conditions and provide a survival strategy for the organism. The bacterial cell wall thickens with cellulose and the bacteria remain in this dormant state until conditions become more favourable. Unlike the *Myxobacteria*, *Vitreoscilla* have not been shown to produce microcysts. Pringsheim detailed seven species of *Vitreoscilla* based on colony morphology and cell dimensions (Pringsheim, 1951). Today three species of *Vitreoscilla* are recognised, *V. beggiatoides* and *V. filiformis* isolated from fresh water sediments and *V. stercoraria* isolated from cow dung. (Holt, Krieg et al., 1994).

Historically *Vitreoscilla* were classified as non-sulfide-oxidising members of the family *Beggiatoaceae* (Holt, Krieg, Sneath, Stanley, and Williams, 1994; Nelson, 1981). In cell morphology and habitat *V. beggiatoides* and *V. filiformis* do resemble the *Beggiatoaceae* (Holt, Krieg, Sneath, Stanley, and Williams, 1994; Nelson, 1981). However, *V. stercoraria* differs in cell shape, habitat and nutritional requirements (Mayfield and Kester, 1975; Nelson, 1981). *V. stercoraria* is the only strain of *Vitreoscilla* that has been kept in pure culture from Pringsheim's original isolation (ATCC 15218) and is the most well characterised of the genus (Brzin, 1966; Costerton, Murray et al., 1961; Mayfield and Kester, 1972; Mayfield and Kester, 1975; Pringsheim, 1951). In 1984 Woese and colleagues carried out 16S ribosomal RNA (oligonucleotide) sequencing on *V. stercoraria* and found it to be closely related to the purple photosynthetic bacteria. *V. stercoraria* was therefore classified in the beta subdivision of the purple bacteria close to species of *Pseudomonas*, *Rhodospirillum*, *Alcaligenes*, *Aquaspirillum*, *Thiobacillus* and *Chromobacterium* (Woese, Weisburg et al., 1984). The grouping of non-photosynthetic bacteria amongst the purple bacteria suggests that a photosynthetic ancestral phenotype gave rise to the greater purple bacterial unit. At various times non-photosynthetic offshoots of the photosynthetic phenotypes arose and it was from one of these that *Vitreoscilla stercoraria* originated (Woese, Weisburg, Paster, Hahn, Tanner, Krieg, Koops, Harms, and Stackebrandt, 1984).

1.1.2 Physiology

In 1972 Mayfield and Kester carried out physiological studies on *V. stercoraria* and investigated a number of characteristics including pH optimum, growth rate, and number of cells per trichome (Mayfield and Kester, 1972). They found that cultures of the organism reached a maximum optical density at 48 hours and

showed no lag phase. There was a marked difference between the number of colony forming units measured by plate count and the optical density measured on a spectrophotometer. This is because long chains of bacterial cells behaved as single colony forming units giving an artificially low count compared to cell numbers indicated by the optical density of the culture. After 15 hours Mayfield & Kester found that the increase in biomass of the culture was because of an increase in trichome length rather than number of trichomes and so the number of colony forming units was no longer rising. The optimum pH for growth of *Vitreoscilla stercoraria* was found to be between 7.5 and 7.7; complete inhibition of the organism was noted below 6.7.

1.1.3 Nutrition

The nutritional requirements of *Vitreoscilla stercoraria* are complex (Mayfield and Kester, 1975). The organism has been shown to require several carbon compounds at substrate levels as carbon and energy sources (Mayfield and Kester, 1975). For example, a requirement for specific amino acids, e.g. tyrosine, has been demonstrated. Potential precursors for these amino acids, e.g. malate, fumarate and succinate, could not substitute for them (Mayfield and Kester, 1975). It was also reported that the organism required acetate in culture media (Costerton, Murray, and Robinow, 1961).

1.1.4 Habitat

Vitreoscilla stercoraria occurs in cow dung (Holt, Krieg, Sneath, Stanley, and Williams, 1994; Reichenbach, 1981). Although isolated from cow dung it is thought that the organism enters the dung from the soil and does not form part of the bovine gut flora (Pengelly and Moody, unpublished observation). It is probable that decomposing material in which ammonification occurs provides the slightly

alkaline pH required by the organism (pH optimum 7.5-7.7) (Mayfield and Kester, 1972). Mayfield and Kester suggest that complex nutritional requirements of *V. stercoraria* may account for its low numbers in soil but they did not take into account that low isolation numbers do not prove low soil numbers.

1.1.5 Motility

All *Vitreoscilla* display gliding motility. This is a trait that they have in common with the *Myxobacteria* and many other bacteria. Studies into the mechanisms involved in gliding motility have been carried out with a number of micro-organisms that use this form of locomotion and various mechanisms have been suggested. None of these studies however have shown conclusively the mechanism involved. It is probable that a number of different mechanisms are used to produce gliding motility and it has been suggested that different mechanisms could even be used by the same organism (Spormann, 1999).

Although many studies into gliding motility have been carried out only Costerton *et al* (Costerton, Murray, and Robinow, 1961) looked specifically at *Vitreoscilla*. They studied two strains of *Vitreoscilla*, both isolated from cow dung, showed that these organisms displayed gliding motility on surfaces but were immobile in fluid suspension. The trichomes were made up of individually motile cells and movement did not arise from a specialised terminal cell or group of cells. Cells could be seen grouping together into longer chains that appeared to move faster than shorter chains (Costerton, Murray, and Robinow, 1961). Costerton *et al*. exposed cells to specific immune serum (antibodies raised against antigens on the cell surface) and observed a cessation of movement suggesting that a superficial structure may be responsible for locomotion. Electron micrographs and various staining techniques have failed to reveal any such surface structures. During

observations of cultures growing on agar Costerton *et al.* reported that trichomes would change direction in order to follow the slime layer left by another trichome and would consequently travel faster than before. They proposed that the slime layer may function as a lubricant to aid in overcoming the forces of surface tension and friction or that it may be necessary for the function of an undiscovered organ of locomotion.

When observed growing on agar it is possible to see the trichomes of *Vitreoscilla* gliding, leaving in their wake a trail of slime that they exude as they move. In 1977 Ridgway proposed that slime extrusion may be the mechanism responsible for gliding in *Flexibacter polymorphus* (Ridgway, 1977). Recently this idea has been given new weight. Hoiczky and Baumeister looked at two species of cyanobacteria using electron microscopy and found pore complexes at the cross walls of the filaments. The pores seem to be involved in slime secretion (Hoiczky and Baumeister, 1998). There is no evidence that *Vitreoscilla* filament walls contain pores. Although many gliding bacteria produce slime it is still not certain whether slime production is the result or cause of gliding motility.

Costerton *et al* reported that the dense outer layer of the cell wall of *Vitreoscilla* included peculiar folding which could also be found in other gliding bacteria including *Myxococcus xanthus*, *Leucothrix* and *Beggiatoa*. They hypothesised that motility could be caused by orderly waves of contraction in the elastic outer layer of the cell wall. This hypothesis would account for gliding motility; the apparent inability of *Vitreoscilla* to swim in fluids; and for the rebound of cells from surfaces that was observed when the cells were grown in liquid media. In 1985 Waterbury and colleagues reported the isolation of strains of cyanobacteria which could swim rapidly in liquid media but lacked flagella (Waterbury, Willey *et al.*, 1985). A

mechanism was proposed in 1996 by Ehlers et al that involved the movement of waves across the surface of the bacterium (Ehlers, Samuel et al., 1996). A wave mechanism could be responsible for the movement of both the cyanobacteria in liquid and gliding bacteria on surfaces but it is unclear as to why this mechanism would allow the cyanobacteria to move in liquid media whilst surface gliding bacteria cannot. When gliding on solid surfaces the bacteria would also have to overcome the problem of adhesion.

The issue of adhesion was touched upon by Keller in 1983 when he looked into a possible mechanism of gliding in Myxobacteria involving surface tension (Keller, Grady et al., 1983). Keller proposed that the production of a surfactant at one end of the bacteria is used to propel the glider up a surface tension gradient. It was postulated by Dworkin in 1983 that the addition of a surfactant such as 0.5 % bovine serum albumin would overcome the gradient and prevent gliding (Dworkin, Keller et al., 1983). This was tested by Burchard when he looked at the effect of surface free energy on gliding by several species of bacteria (Burchard, 1986). Only *Oscillatoria princeps* was unaffected by the addition of a surfactant. Burchard found that the mechanism of gliding was not affected by surfactants but that the explanation for the inability of gliders to move on a surface with low surface free energy is a problem with adhesion. Further evidence for the importance of adhesion came from observations on species of *Cytophaga*, *Flexibacter*, *Microscilla* and *Myxococcus* (Burchard, Rittschof et al., 1990). The observation that the bacteria adhered more tenaciously to hydrophobic surfaces than hydrophilic ones suggested that the bacterial component that makes contact with the surface is relatively hydrophobic. Adhesion may be mediated by a water exclusion mechanism. On surfaces that are very hydrophobic water may be so successfully excluded that the level of adhesion is such that the bacteria are

unable to move. On hydrophilic substrata water exclusion is poor and close contact may be prevented, again accounting for limited movement. The necessity for adhesion may also explain why specific immune antiserum prevents gliding motility. It may interfere with the cells' adhesion to the surface rather than by blocking a specific locomotion organ as Costerton *et al* suggested.

The effect of nutrient levels on colony morphology of gliding bacteria has been studied in a number of bacteria. The formation of spreading colonies of gliding bacteria has been shown to be favoured by low nutrient concentrations; when high nutrient levels were used colonies tended to be raised and smooth-edged (Burchard, 1981; Duxbury, Humphery *et al.*, 1980; Wolkins and Pate, 1984). In 1993 Gorski *et al.* looked at the inhibition of gliding motility of *Cytophaga johnsonae* by sugars. Gorski *et al.* found that sugars with a common structure inhibited the motility of the organism. They found that if any of the substituents on carbons 3, 4, 5, or 6 of a glucoside were altered then the compound had little or no effect on gliding. The effect was found to be completely reversible and Gorski *et al* considered it possible that the sugars were binding to an enzyme or regulatory protein on the cell surface that is involved in the control of motility. It is possible however that the sugars affected the hydrophobicity of the surface and so, as Burchard suggested, affect the ability of the organism to adhere to the surface. The effect of sugars on gliding motility was used by Xianzhen Li and Feng Chen to improve isolation methods for cellulolytic gliding bacteria (Li and Chen, 1998). They found that the elimination of soluble sugars from media not only inhibited contaminating fungi but also stimulated gliding making the gliding bacteria easier to isolate from other microorganisms.

Several studies of gliding motility have involved the attachment of beads to the surface of the organism and then observing the movement of the beads. Lapidus and Berg attached beads to the surface of a *Cytophaga* and observed their movement (Lapidus and Berg, 1982). They found that the beads moved around with or against the direction of movement of the cell and that movement of both the beads and the cell stopped as oxygen became depleted. Beatson and Marshall used this approach and proposed a helical mechanism of gliding motion based on observations of spheres that they attached to the surface of a typical species of *Cytophaga* and two unclassified gliding bacteria (Beatson and Marshall, 1993). Microscopic spheres attached to the bacteria moved in helical paths around them. The mechanism is proposed to be oriented helically on the cell surface and acts on a substratum via an adhesive polymer network. Spormann and Kaiser used a similar technique on *Myxococcus xanthus* (Spormann and Kaiser, 1995). They found that beads may move forwards and backwards whilst the cell is moving forwards and that the beads can move whilst the cell is stationary. It was also noted that two beads on the same cell could move in opposite directions at the same time. Beads tended to become trapped at the ends of the cell. Similar studies on *Flexibacter polymorphus* and *Flexibacter johnsoni* have shown bead movement of a similar type (Gorski, Leadbetter et al., 1991; Ridgway and Lewin, 1988). In all bacteria so far examined the speed of bead movement was similar to gliding speed suggesting that the movement of the beads is produced by the same mechanism.

None of the proposed mechanisms to explain the movement of gliding bacteria so far have been conclusively proved. It seems likely that elements of each proposal will prove to be relevant. Different mechanisms may be employed by different organisms. Some bacteria may even employ more than one system in common

with the myxobacteria, which have been shown to employ two different systems of locomotion.

1.2 Electron transport chains

1.2.1 Types of carrier

In the course of the oxidation of highly reduced organic compounds electrons are removed and passed along a chain of carrier molecules termed the electron transport chain. The final electron acceptor is usually oxygen, although some microorganisms use an organic compound such as fumarate or an inorganic compound such as nitrate. Passing electrons along a chain of carriers releases energy which is conserved and used to synthesise ATP.

Electron transport chains in all organisms show many similarities. Mitochondria possess a relatively simple electron transport chain with electrons passing to ubiquinol via complex I, II or electron transferring flavoprotein but from this point the chain is linear, with only one terminal oxidase. This contrasts with many bacterial chains, which are branched and may possess two or more terminal acceptors. In mitochondria electrons are transported down the respiratory chain through a redox potential span of 1.14 volts, from the NAD^+/NADH couple to the $\text{O}_2/2\text{H}_2\text{O}$ couple (Nicholls and Ferguson, 1992).

In mitochondria the main components of the electron transport chain are present as four membrane bound complexes and two mobile carriers, coenzyme Q and cytochrome C. Electron carrying molecules within mitochondria can be divided into six main types: NAD^+/NADH , flavins, non haem iron (iron-sulphur centers), quinones, protein-bound copper and haem iron. The active part of NAD^+ is the nicotinamide (or pyridine) residue, which can accept another hydrogen atom at position 4 on its ring. Nicotinamide is the vitamin niacin. NAD^+ and NADH both

absorb ultraviolet light at 260 nm; this is because of their purine ring structure. NADH has an absorbance peak at 340 nm while NAD^+ does not absorb at this wavelength; this can be used to monitor reactions catalysed by pyridine-nucleotide-linked dehydrogenases or reactions that can be linked to these dehydrogenases. The disappearance or appearance of NADH can be monitored spectrophotometrically (Nicholls and Ferguson, 1992).

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are prosthetic groups contained in flavoproteins that are also oxidoreductases. There are four main flavoproteins bound to the mitochondrial inner membrane and involved in electron transport. NADH dehydrogenase which contains FMN, succinate dehydrogenase which contains FAD, glycerol-3-phosphate dehydrogenase containing FAD and electron transferring flavoprotein (ETF) which contains FAD. All these proteins (except ETF) contain non-haem iron associated with acid-labile sulphur (yields H_2S when treated with acid). Unlike NAD^+ in pyridine-nucleotide-linked dehydrogenases FAD and FMN in the flavin-linked dehydrogenases are tightly bound to the protein part of the enzyme. Oxidation and reduction in flavoproteins takes place in the isoalloxazine ring system, which is capable of accepting 2 hydrogens from the substrate. FAD is derived from vitamin B_2 (riboflavin) and as with NAD^+ its oxidation/reduction can be monitored spectrophotometrically because FAD has an absorption peak at 450 nm, which disappears on reduction to FADH_2 (Nicholls and Ferguson, 1992).

Iron-sulphur centres consist of iron atoms covalently bound to proteins by cysteine sulphurs and to other atoms by sulphur bridges. There are 10 iron-sulphur centres associated with mitochondria. Oxidation and reduction is between Fe(II) and Fe(III) . Fe centres may contain two or four Fe atoms; despite this each centre only

acts as a one-electron carrier. Iron-sulphur proteins are widely distributed amongst electron transport chains and have widely different redox midpoint potentials. Quinones are lipid soluble hydrogen carriers in the membrane. They have substituted benzoquinone with a polyisoprene side chain. This chain can contain between 0 and 10 isoprene units; ubiquinone found in mitochondria has 10. Oxidation/reduction occurs in the benzoquinone nucleus, an absorbance band with a maximum at 275 nm disappears when the molecule is reduced.

Haem iron is a carrier that is a prosthetic group in cytochromes. The basis of haem is porphyrin, which consists of four pyrrole rings linked in a cyclic manner by methane bridges. Various groups are attached to the porphyrin nucleus to give porphyrins. The most common porphyrin in biological systems is protoporphyrin IX. Haem is a porphyrin ring in which the four pyrrole nitrogens are coordinated to a iron atom forming a square planar complex. Positions 5 and 6 of the iron's 6 coordination positions, which are perpendicular to the plane of the porphyrin ring, are occupied by sidechains of specific amino acids from the protein.

Cytochromes were originally classified according to the position of the alpha absorption band of their haems. Haems are classified into three types *a*, *b* and *c* according to substituents bound to the porphyrin. Type *a* cytochromes contain haem A. Type *b* contain protohaem and type *c* contain haem C covalently bound to the protein. When oxidised, cytochromes have two peaks in the visible range and when reduced they have three (α , β and γ). Absolute spectra however are of limited use because of non-specific absorption and light scattering. Therefore the oxidised spectrum is subtracted from the reduced spectrum to give the difference spectrum. Individual cytochromes are most easily resolved using the α -absorption

bands in the 550-610 nm region. Room temperature spectroscopy can only distinguish between single *a*-, *b*- and *c*-type cytochromes. However each type can be further subdivided into two spectrally distinct components. Type *a* cytochromes can be divided into *a* and *a*₃. These two subtypes can be distinguished using CO, which binds specifically with *a*₃. *a* and *a*₃ are identical but are bound in different environments. The type *b* cytochromes consist of two components with different *E*_m values (high *b*_h and low *b*_l). The two subtypes reflect the presence on one polypeptide chain of two *b*-type haems; the different local environments provided by the polypeptide chain account for the differences in spectral and redox properties. Low temperature spectroscopy can resolve the two *c* type cytochromes (cyt *c* and cyt *c*₁). Cyt *c*₁ is an integral protein within complex III while cyt *c* is a peripheral protein on the positive side of the membrane to which protons are pumped and links complex III with cytochrome *c* oxidase (Nicholls and Ferguson, 1992).

Copper is also utilised in the components of the electron transport chain. The mammalian mitochondrial cytochrome *c* oxidase contains haems *a* and *a*₃ together with at least two copper atoms termed Cu_A and Cu_B. Electrons from cyt *c* are initially transferred to Cu_A. The other redox components (Cu_B and haem *a*₃) are located close to each other and form a binuclear centre located towards the negative side of the membrane (Nicholls and Ferguson, 1992).

Electron carrying molecules within the mitochondria are organised into four complexes and two mobile carriers (see Figure 1.1 Page 15). Complex I (NADH -UQ oxidoreductase) contains more than thirty polypeptides. The complex catalyses the transfer of two electrons from NADH to ubiquinone in a reaction that is associated with proton translocation across the membrane. Apart from one

molecule of the flavin FMN the redox centres of complex I are iron sulphur centres and the complex contains up to seven of them. It is possible to inhibit complex I with either rotenone or piericidin A.

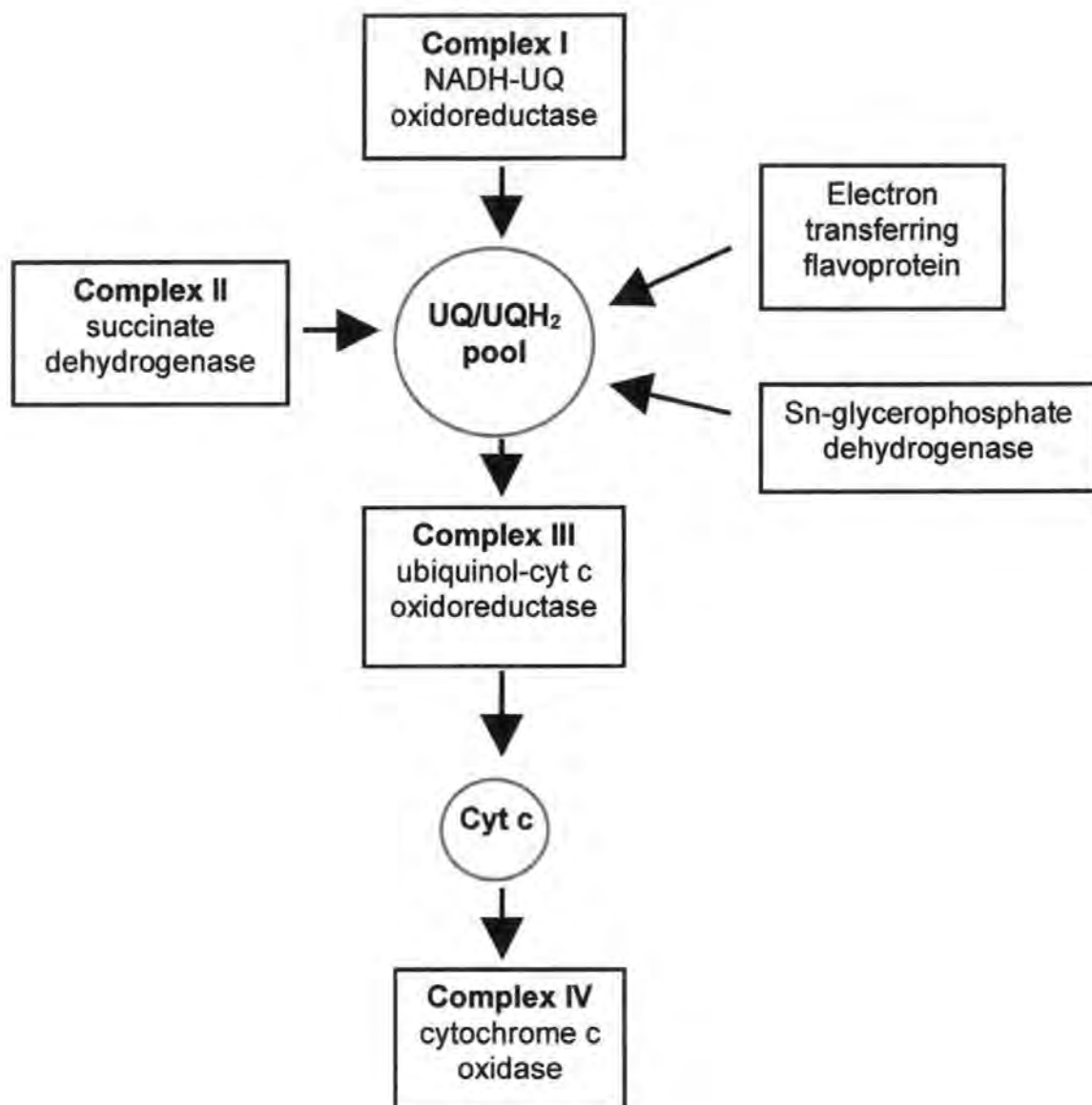


Figure 1. 1 An overview of the mitochondrial respiratory chain

There are three other redox pathways which feed into ubiquinol: Complex II, electron transferring flavoprotein and *sn*-glycerophosphate dehydrogenase. Electron transferring flavoprotein transfers electrons from the flavoprotein-linked step of fatty acid β -oxidation and like complex II is located on the matrix face of the membrane. Complex II transfers electrons from succinate and consists of several polypeptides. The two largest polypeptides constitute succinate dehydrogenase, with the largest containing a covalently bound FAD and two Fe/S centres. The other polypeptide contains another Fe/S centre. Complex II also contains a haem *b* of unknown function that is associated with the smaller polypeptides.

Complex III is also termed cyt *bc*₁ or ubiquinol-cytochrome *c* oxidoreductase and transfers electrons from ubiquinol to cyt *c* as well as translocating protons across the membrane. This complex is also found in many bacteria and is similar to the plastoquinol-plastocyanin oxidoreductase (cyt *bf* complex) of thylakoids. Cytochrome *bc*₁ complexes contain three polypeptide chains that carry the redox groups: an iron-sulphur protein, cyt *c*₁ and cyt *b*. The iron-sulphur protein contains a 2Fe/2S cluster attached to the polypeptide by chelation of one Fe to two cysteines and the other to two histidine residues. The iron-sulphur protein is anchored to the membrane by a hydrophobic N-terminus. Cytochrome *c*₁ has a similar structure to the iron-sulphur protein except it is the C-terminus which provides the hydrophobic anchor. The cyt *b* subunit binds two haem groups which have axial histidine ligands.

Complex III transfers electrons to cytochrome *c*. Cytochrome *c* is a peripheral protein located on the outer face of the mitochondrial membrane and is easily solubilised from intact mitochondria. The haem is located in a largely hydrophobic crevice with only one edge exposed to solvent.

1.2.2 Bacterial electron transport

Bacteria use many different sources and acceptors of electrons. Components of the electron transport chain differ not only between different organisms but also in the same organism depending on growth conditions. Bacterial electron transport chains are generally more branched than mitochondrial chains. There can be several dehydrogenases, which feed into the chain, and two or three terminal oxidases, which can each, reduce molecular oxygen to water. The ratio of terminal oxidases is determined by growth conditions.

The soil organism *Paracoccus denitrificans* has an electron transport chain whose components are similar to the components of the mitochondrial system. There is however a difference in the number of polypeptide chains that complexes II, III and IV contain. The *P. denitrificans* counterpart to complex I has not yet been isolated although antibody cross-reactivity and some gene sequence data indicate that it is closely related to the mitochondrial complex I (Xu, Matsuno-Yagi et al., 1991). Cytochrome c_{550} from *P. denitrificans* is closely related to mitochondrial cytochrome *c* in terms of both structure and redox potential. It would therefore be logical to presume that c_{550} shuttles between proteins which correspond to the bc_1 and aa_3 complexes; however a membrane bound c_{552} may instead carry out this role. Deletion of the gene for cytochrome c_{550} does not stop electron transfer to cytochrome aa_3 . (Nicholls and Ferguson, 1992). In *P. denitrificans* cytochrome aa_3 does not provide the only route to oxygen; there is an alternative that bypasses both bc_1 and aa_3 and probably a third oxidase that can accept electrons from *c*-type cytochromes. *P. denitrificans* is also able to utilise terminal acceptors other than oxygen. For example H_2O_2 which is commonly found in soil can be used.

The reduction of H_2O_2 is catalysed by a periplasmic cytochrome-c peroxidase which is a dihaem c-type cytochrome (Nicholls and Ferguson, 1992).

The aerobic electron transport chain of *E. coli* varies considerably from mammalian mitochondria or *Paracoccus denitrificans*. *E. coli* does not possess c-type cytochromes or a bc_1 complex (see Figure 1.2 Page 19). Evidence for the lack of a bc_1 complex comes from the insensitivity of electron transport between ubiquinol and oxygen to antimycin and myxothiazol, which are inhibitors of the bc_1 complex (Nicholls and Ferguson, 1992). *E. coli* possesses two oxidases (cytochromes *bo* and *bd*) that directly oxidise ubiquinol.

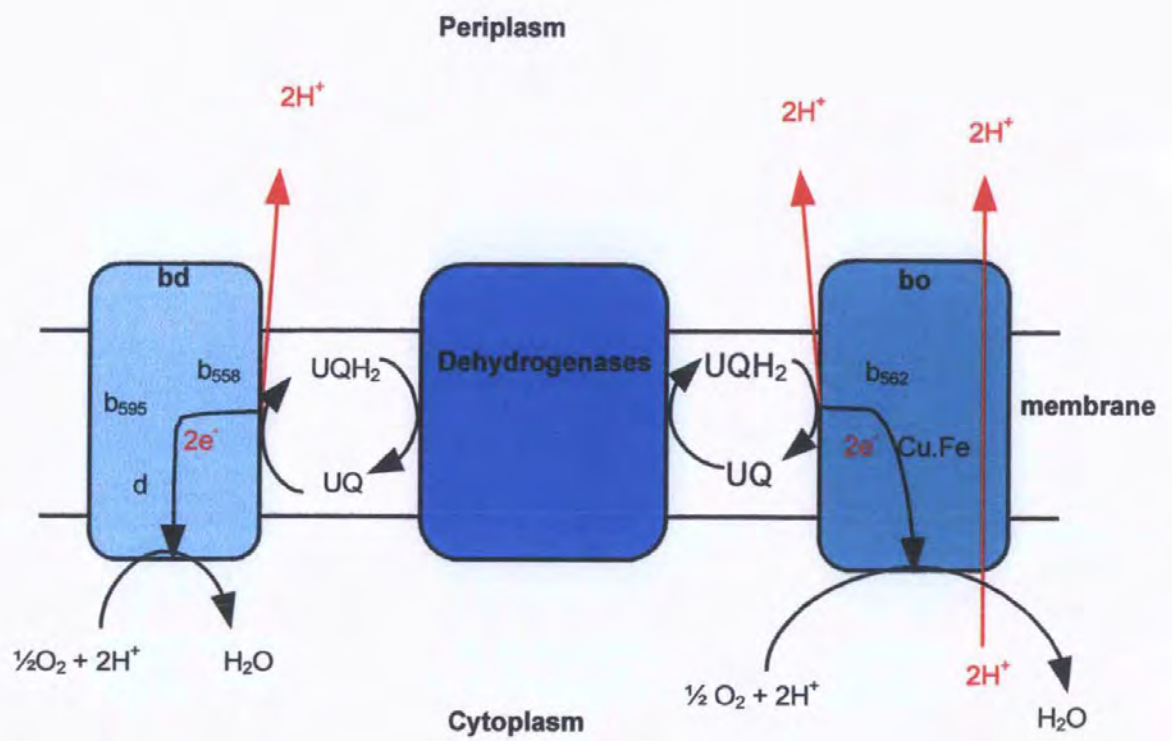


Figure 1. 2 The respiratory chain of *Escherichia coli*

Cytochrome *bo* from *E. coli* is a four subunit haem-copper oxidase that catalyses the four electron reduction of O₂ to water and is also a proton pump (Puustinen, Finel et al., 1991). All of the redox centres are located in subunit I which is the largest of the subunits. A low spin protohaem (haem *b*) acts as the electron donor to a binuclear centre that is composed of an O-type haem (haem O₃) and a copper ion (Cu_b). Subunits I, II and III of ubiquinol oxidase are the same as the corresponding subunits in the *aa*₃-type cytochrome *c* oxidases whilst subunit IV has little sequence homology with other oxidases and its function is unknown. Unlike cytochrome-*c* oxidases, subunit II of ubiquinol oxidase has no Cu_A centre (Puustinen, Finel, Haltia, Gennis, and Wikström, 1991), nor does it have a cytochrome *c* binding site (Lemieux, Calhoun et al., 1992; Nicholls and Ferguson, 1992). The haem *b* receives electrons directly from a membrane solubilised ubiquinol molecule and the resulting protons are released on the positive side of the membrane (Puustinen, Finel, Haltia, Gennis, and Wikström, 1991).

Cytochrome *bd* is the only well characterised bacterial terminal oxidase, that is unrelated to the haem-copper oxidase superfamily. It has been suggested that in *E. coli* and other enteric bacteria, cytochrome *bd* serves as a high affinity oxidase to support energy-requiring processes under microaerophilic conditions and to protect anaerobic processes from inhibition from oxygen (Jünemann, 1997; Lemieux, Calhoun, Thomas, Ingledew, and Gennis, 1992). Loss of *bd* in *E. coli* leads to a lowered ability of the organism to cope with oxidative stress (Jünemann, 1997; Lemieux, Calhoun, Thomas, Ingledew, and Gennis, 1992). Cytochrome *bd* is made up of two polypeptide chains and contains two B-type haems as well as the distinctive porphyrin ring of the D-type haem, the site of oxygen reduction.

1.2.3 Electron transport in *Vitreoscilla*

In common with *E. coli*, *Vitreoscilla* contains both cytochrome *bo* and *bd* terminal oxidases in its electron transport chain and quinol is the direct electron donor to the terminal oxidase (Georgiou and Webster, 1987a; Georgiou and Webster, 1987b). Cytochrome *bo* from *Vitreoscilla* has been purified and partially characterised and has been found to be structurally and functionally similar to cytochrome *bo* from *E. coli* (Georgiou, Cokic et al., 1988). Both enzymes have four subunits of similar corresponding weights and both contain haem type *b* and Cu^{2+} prosthetic groups. The enzymes also have similar optical spectra and are inhibited by KCN and sodium azide. Both also exhibit ubiquinol-1 oxidase activity (Georgiou, Cokic, Carter, Webster, and Gennis, 1988).

Vitreoscilla differs from *E. coli* in that *Vitreoscilla* produces high levels of haemoglobin (VHb) in its cytoplasm under hypoxic conditions. Although several bacteria and yeast produce a related protein, termed flavohemoprotein, *Vitreoscilla* is the only bacterium known to produce this form of haemoglobin. *Vitreoscilla* haemoglobin is thought to capture oxygen and feed it into the terminal oxidase under oxygen limiting conditions (Wakabayashi, Matsubara et al., 1986); an increase of the haem content in the cell has been observed when the oxygen concentration in the growth medium fell (Boerman and Webster, 1982). The gene encoding for VHb (*vgb*) has been identified, cloned and expressed in *E. coli*. The resulting strain of *E. coli* grew better than the *E. coli* controls under microaerobic conditions (Khosla and Bailey, 1989) (Dikshit, Dikshit et al., 1990).

It was originally thought that VHb was a soluble terminal oxidase (cytochrome *o*). This was because VHb is more autoxidisable than other haemoglobins, cytochrome *o* and VHb are spectroscopically similar (the difference between the

principle Soret bands in the CO difference spectrum is only 3 nm) and the oxidation-reduction turnover of VHb is very slow in the presence of NADH-metHb reductase (Webster and Liu, 1974) (Tyree and Webster, 1978). However, the true nature of this molecule became apparent when the gene was sequenced and homology with eukaryotic haemoglobins was discovered (Wakabayashi, Matsubara, and Webster, 1986). Other properties of VHb also pointed to its similarity with eukaryotic haemoglobin. Firstly VHb was isolated from the soluble fraction of the cell, and secondly the infrared spectrum of the oxygenated form of VHb is similar to those of oxymyoglobins and oxyhaemoglobins (Choc, Webster et al., 1982). *Vitreoscilla* VHb is a homodimeric protein with a molecular mass of 32,783 Da. Each subunit has one protohaem IX (Tyree and Webster, 1978), (Webster and Oriei, 1985), (Wakabayashi, Matsubara, and Webster, 1986). If the ferrous form of VHb is bound to CO and O₂ it forms compounds which are optically very similar to those of myoglobin and haemoglobin (Webster and Liu, 1974). Rapid kinetic studies of the binding of CO and O₂ to VHb have shown it to have a relatively open haem pocket. Oriei and Webster propose that this may explain why VHb is more autooxidisable than other oxyhaemoglobins (Webster and Oriei, 1985).

1.2.4 Sodium pumps

According to the chemiosmotic hypothesis (Mitchell, 1961) energy for ATP synthesis is harnessed by the establishment of a proton gradient across an energy-transducing membrane. This is accomplished by the use of proton pumps that are coupled to the electron transport chain and hydrogen gradient driven ATP synthetase. However, in 1981 Tokuda and Unemoto showed that the marine bacterium *Vibrio alginolyticus* (*V. alginolyticus*) possesses a primary Na⁺ pump that is directly coupled to its respiratory chain (Tokuda and Unemoto, 1981). They later found that the NADH dehydrogenase activity of *V. alginolyticus* required Na⁺

for maximum activity and that activity of a mutant that lacked the Na^+ pump was not stimulated by Na^+ (Tokuda and Unemoto, 1984). *V. alginolyticus* is not the only marine bacterium to have been found to contain a primary Na^+ pump. Other marine bacteria that have proved to pump Na^+ include a halotolerant Ba_1 (Kendror, Preger et al., 1986), the halotolerant marine bacterium *Alcaligenes* strain 201 (Oh, Kogure et al., 1991), *Vibrio parahaemolyticus* (Tsuchiya and Shinoda, 1985) and *Vibrio costicola* (Udagawa, Unemoto et al., 1986). A similar pump has also been detected in *Klebsiella pneumoniae* (Dimroth and Thomer, 1989). All the above sodium pumping bacteria extrude Na^+ at the NADH:quinone oxidoreductase segment in the respiratory chain. All also require Na^+ for maximum activity and can be inhibited by the addition of 2-heptyl-4-hydroxyquinoline N-oxide.

The most well studied example of a primary sodium pump from a marine bacterium is that of *V. alginolyticus*. Two NADH:ubiquinone oxidoreductases have been found in the membrane of *V. alginolyticus* (Hayashi and Unemoto 1987, Hayashi et al 1992). One of these is of the non-energy conserving type whereas the other is believed to be coupled to Na^+ ion pumping. This enzyme is induced during aerobic growth at alkaline pH and may have the physiological advantage of keeping the cytoplasmic pH near neutrality while pumping cations (Na^+) into an alkaline environment (Tokuda and Unemoto, 1981). Analysis of the Na^+ pump has revealed FAD but no FMN, non-haem iron, an acid labile sulphur cluster and tightly bound ubiquinone-8 (PfenningerLi, Albracht et al., 1996). The participation of an iron-sulphur cluster of the 2Fe-2s type in electron translocation was demonstrated by Pfenninger Li.

When Na^+ is not present the path of electrons ends with the reduction of ubiquinone-1 to the semiquinone derivative which, in the presence of oxygen,

becomes reoxidised with accompanying formation of superoxide radicals. With Na^+ present these oxygen radicals are not formed and the semiquinone is further reduced to the quinol derivative, indicating that the Na^+ dependant step catalysed by NADH:ubiquinone oxidoreductase is the reduction of ubisemiquinone to ubiquinol (PfenningerLi, Albracht, van Belzen, and Dimroth, 1996). Pfenninger *et al* also reconstituted the enzyme into liposomes and showed that NADH oxidation by ubiquinone-1 was coupled to Na^+ transport with a stoichiometry of 0.5 Na^+ per NADH oxidised. With the use of inhibitors he also showed that Na^+ transport is a primary event and does not involve the intermediate formation of a proton gradient.

Sodium ion coupled oxidative phosphorylation has been demonstrated in *V. alginolyticus* (Dibrov, Lazarova *et al.*, 1986). It had been shown that the sodium gradient produced can be used for all three types of membrane-linked work. Dibrov *et al.* demonstrated chemical work in the form of ATP synthesis (Dibrov, Lazarova, Skulachev, and Verkhovskaya, 1986). Dibrov *et al* also showed that the gradient could be used for mechanical work when he showed that it is used to drive the rotation of flagellum (Dibrov, Kostyrko *et al.*, 1986). Tokuda & Unemoto showed the use of the sodium gradient to drive osmotic work by demonstrating the uphill import of metabolites (Tokuda and Unemoto, 1982).

The strict aerobes *Propionigenium modestum* and *Veillonella alcalescens* have both been shown to utilise a sodium pumping methylmalonyl-CoA decarboxylase (Hilpert, Schink *et al.*, 1984; Hilpert and Dimroth, 1982). Both organisms ferment succinate to propionate and CO_2 via succinyl-CoA, (R) and (S)-methylmalonyl-CoA and propionyl-CoA. Only one reaction in this pathway (decarboxylation of (S)-methylmalonyl-CoA to propionyl-CoA) generates sufficient energy for energy

conservation (Hilpert, Schink, and Dimroth, 1984). The enzyme is membrane bound and biotin-containing and is coupled to the electrogenic extrusion of two Na^+ per reaction (Hilpert, Schink, and Dimroth, 1984; Hilpert and Dimroth, 1984; Dimroth, 1987). *P. modestum* has been shown to utilise the Na^+ gradient created by its Na^+ pumping methylmalonyl-CoA decarboxylase with a Na^+ dependant ATPase (Hilpert, Schink, and Dimroth, 1984).

There have been reports that alkotolerant/halotolerant strain of *Bacillus* (Semeykina, Skulachev et al., 1989) and *E. coli* grown at alkaline pH (Avetisyan, Dibrov et al., 1989) utilise a sodium pumping terminal oxidase. Neither report identified the enzyme responsible and have to date not been expanded upon. *Vitreoscilla* is therefore the only bacterium that has been reported to possess a sodium pumping terminal oxidase that has been identified and partially characterised (Bassey, Efiok et al., 1990; Bassey, Efiok et al., 1992; Bassey, Efiok, and Webster, 1990; Park, Moon et al., 1996). In 1990 Bassey *et al* first reported the generation of a sodium gradient by the respiratory chain of *Vitreoscilla* (Bassey, Efiok, and Webster, 1990). Bassey *et al* demonstrated that the formation of $\Delta\psi$ was directly coupled to respiratory-driven Na^+ extrusion by putting forward the following experimental evidence: there was a correlation between the kinetics of respiratory-driven Na^+ extrusion and $\Delta\psi$ formation; monensin caused the collapse of $\Delta\psi$; the protonophore 3,5-di-tert-butyl-4-hydroxybenzaldehyde (DTHB) caused a transient collapse followed by the stimulation of Na^+ extrusion and recovery of the $\Delta\psi$, and there was no correlation between $\Delta\psi$ and H^+ fluxes (Bassey, Efiok, and Webster, 1990).

Further studies of the respiratory chain of *Vitreoscilla* by Bassey *et al.* revealed that the terminal oxidase was responsible for the sodium pumping (Bassey, Efiok,

and Webster, 1990). Bassey *et al* initially looked at two possible candidates for the sodium pump, an NADH dehydrogenase as found in the marine bacteria or cytochrome *bo* (the terminal oxidase). They found that although the NADH oxidase activity of the membranes was enhanced more by Na^+ than Li^+ the quinol oxidase activity was only enhanced by Na^+ . When the cytochrome was initially reconstituted into liposomes derived from *E. coli* phospholipids movement of Na^+ both in and out of the liposomes (depending on Na^+ concentration inside and outside) was detected, and the enzyme catalysed a net uptake of $^{22}\text{Na}^+$ when the liposomes were energised with ascorbate/ N,N,N',N'-tetramethyl-1,4-phenylenediamine (TMPD) thus adding to the evidence that the terminal oxidase from *Vitreoscilla* is responsible for pumping sodium (Bassey, Efiok, and Webster, 1990). Further studies by Park *et al* in which the enzyme was incorporated into liposomes made from *Vitreoscilla* phospholipids saw an increase in extrusion of Na^+ compared with the *E. coli* liposomes (Park, Moon, Cokic, and Webster, 1996). They found an efficiency of 3.93 Na^+ pumped per O_2 consumed when ascorbate/TMPD was used as the substrate.

In 1991 Bassey looked at the synthesis of ATP by *Vitreoscilla* and reported the use of a sodium gradient to generate ATP (Bassey, Efiok, and Webster, 1992). They showed that the amount of ATP generated was dependent on the magnitude of the Na^+ gradient imposed and that anaerobic cells which had been equilibrated with Na^+ were able to synthesise sufficient ATP to double the intracellular concentration when they were exposed to O_2 . From this evidence Bassey *et al* reported that under normal growth conditions Na^+ is probably the main coupling ion for ATP synthesis in *Vitreoscilla*.

1.3 Initial aims and objectives

1.3.1 Aims

1. Isolation of *Vitreoscilla* from the environment.
2. Extraction of membranes containing cytochrome *bo* from *Vitreoscilla*.
3. Purification of cytochrome *bo* from membranes.
4. Reconstitution of the purified enzyme into phospholipid vesicles with defined sidedness, i.e. where the orientation of the enzymes in the artificial membrane is known and is as uniform as possible.
5. Unequivocal determination of the ion-pumping capacity of the enzyme.
6. Development of methods for the deconvolution of the contributions of enzyme intermediates to the electronic absorption spectrum during turnover.
7. Development of a kinetic scheme that links these enzyme intermediates and that incorporates the effects of pH and the concentration of sodium.
8. Identification of further examples of bacteria with sodium-pumping oxidises.

1.3.2 Objectives

1. Establishment of preparative methods: medium-scale growth of bacteria that require high aeration levels; isolation of bacterial membranes; purification of cytochromes *bo* from *Vitreoscilla* and *E. coli* RG145 using methods that are as similar as possible; and initial reconstitution studies.
2. Measurement of enzyme-induced Na^+ and H^+ fluxes using both ion-selective electrodes and spectrophotometric methods, with the H^+ pumping cytochrome *bo* from RG145 as a control.
3. Characterisation of the electronic absorbance spectra of intermediates in the turnover of *Vitreoscilla* cytochrome *bo*.
4. Determination of the population of the enzyme intermediates during turnover and of the effects of pH and Na^+ concentration.
5. Screening of sediment and water column samples taken from marine, estuarine and ruminant faecal sources for Na^+ dependant growth.
6. Identification and medium-scale growth of isolates; preparation of bacterial membranes; and preliminary screening using electronic absorbance spectroscopy to determine their cytochrome complement.
7. Characterisation of the respiratory chains of promising isolates and preliminary purification of enzymes of interest at least as far as solubilisation.

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Chapter 2

The isolation, purification and characterisation of *Vitreoscilla stercoraria* strains from the environment

2.1 Introduction

Vitreoscilla have been found in cow dung, fresh water sediments, rotting vegetation and stagnant ponds (Pringsheim, 1951) (Costerton, Murray et al., 1961). Webster's original strain that was proposed to pump Na^+ was isolated from cow dung (Bassey, Efiok et al., 1990). It is therefore probable that it was a strain of *Vitreoscilla stercoraria* (the only species reportedly isolated from cow dung) (Holt, Krieg et al., 1994). Cow dung was therefore sampled for a strain of *Vitreoscilla stercoraria* that might also possess a sodium pumping terminal oxidase. The ease of sampling and straight forward recovery techniques also made sampling from cow dung the preferred option. Other dung samples (sheep and horse) were also taken and some qualitative comparisons made of numbers of possible *Vitreoscilla* strains isolated from each type of dung. Dartmoor was chosen as the sample site because of its proximity to the university. Dartmoor also has good access and numerous cattle graze freely on the moor in the summer.

Pringsheim described a method for the isolation of *Vitreoscilla* from cow dung in which a small quantity of dung is placed on the centre of an agar plate. Gliding bacteria will move away from the central inoculation and it is then possible to pick them off the plate (Pringsheim, 1951). Costerton *et al* used a similar method but streaked the dung onto the plate (Costerton, Murray, and Robinow, 1961).

The aims of the work presented in this chapter were to isolate, purify and characterise *Vitreoscilla* strains from the environment. At the outset of the study Dr Dale Webster had supplied a strain of *Vitreoscilla* from his laboratory (Dr D Webster Illinois Institute of Technology, Chicago, Illinois, 60616). This was the

strain that had been reported to possess a sodium dependent terminal oxidase (Bassey, Efiok, and Webster, 1990; Bassey, Efiok et al., 1992). Instructions were supplied for the growth of the organism including a recipe for agar based on yeast extract, peptone and salt free acid digest of casein. Instructions were also received concerning frequency of subculture of the organism from the stock. In Dr Webster's lab the organism was kept on agar slopes at 4°C and subcultured onto fresh agar every few months. Dr Webster did not mention how often the organism was retrieved from an original stock. When this strain was recovered from storage and grown on agar the bacteria no longer displayed gliding motility. It is probable that repeated subculture in Dr Webster's laboratory had resulted in changes to some of the characteristics of the organism. The organism had been grown on agar that did not contain any added sodium ions and so it is possible that the sodium-dependent trait could have been lost. Initial papers on the organism also reported that it required acetate to grow (Costerton, Murray, and Robinow, 1961); there was no acetate in the recipe received from Dr Webster and later papers make no mention of the requirement. As it was not possible to determine the extent to which the bacteria had changed, it was thought prudent not to use it in the study. It was therefore decided to isolate a *Vitreoscilla* strain directly from the environment.

The bacteria isolated from dung were characterised. Characteristics such as cell width, motility, the affinity of the organism for sodium chloride and the effect of potassium on growth were investigated. The effect of monensin, a Na⁺ specific ionophore that catalyses Na⁺/H⁺ exchange across membranes and FCCP, an uncoupler of oxidative phosphorylation were also investigated.

2.2 Materials and methods

2.2.1 Reagents

Agar technical number 3, Tryptone (L42), and yeast extract (L21) were supplied by Oxoid. (Basingstoke, Hampshire) All other reagents were supplied by Sigma (Poole, Dorset) unless otherwise stated.

2.2.2 Isolation

Vitreoscilla were isolated on a modified tryptone, yeast (MTY) agar with added sodium chloride. This consisted of 12 g l⁻¹ of agar, 10 g l⁻¹ yeast extract, 10 g l⁻¹ of tryptone and 2.5 g l⁻¹ of sodium chloride. The pH was adjusted to 7.8 with sodium hydroxide. Liquid cultures were grown in MTY broth. The broth contained the same ingredients as the agar but without the agar.

Samples of cow dung (both fresh and older deposits) along with horse and sheep droppings were collected from a site near Cadover Bridge on Dartmoor, Devon (grid reference 5555 6465). A pea-sized quantity of each sample was placed in the centre of five agar plates. The plates were incubated at 25° for 12 hours and then examined for growth under a low powered microscope. Bacteria that had moved away from the central inoculum were picked off and streaked onto fresh plates. The plates were incubated for a further 12 hours and then re-examined. Bacteria moving away from the central inoculation were again picked off and streaked onto fresh plates.

2.2.3 Purification of cultures

Two loops of contaminated bacteria were added to 1ml of phosphate buffered saline (Fisher Scientific Ltd, Loughborough, Leicestershire) containing 0.005 g l⁻¹

lysozyme (from chicken egg white). The saline suspension was shaken for 10 seconds, left for 15 minutes at room temperature then shaken again for 10 seconds. A loop of the suspension was streaked onto MTY agar and the plates were incubated for 12 hours at 25°C. The single colonies suspected of being *Vitreoscilla* were picked off under a low powered microscope with a flattened loop and streaked onto MTY agar.

2.2.4 Culture conditions

Unless otherwise stated *Vitreoscilla* were grown on MTY agar or in MTY broth. Agar plates were streaked or spread on the day they were poured without drying. An overnight culture is defined as a single colony from an agar plate, inoculated into broth and incubated for 48 hours at 25 °C without shaking.

2.3 Results

2.3.1 Isolation

Three trips were made to Dartmoor to collect dung samples. As well as cow dung, sheep and horse dung samples were also collected. Six isolates were recovered during the first visit on the 25/2/98; none of these proved to be species of *Vitreoscilla*. The second visit (21/8/98) yielded 10 possible *Vitreoscilla* strains of which 6 were confirmed. The final visit was on the 16/10/98 and two isolates were retained, neither of these proved to be *Vitreoscilla* species. Figure 2.1 (Page 41) shows an isolation plate with gliding bacteria moving away from the central inoculation of dung. Table 2.1 (Page 42) shows the details of the bacteria isolated from various sources. The results of the Gram stain, gliding observation and whether or not the cells grew in chains are all shown. This information was used to identify possible strains of *Vitreoscilla* for further work.

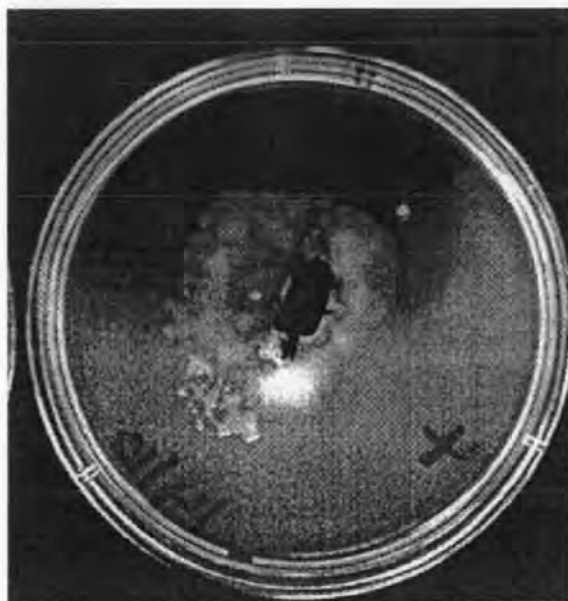


Figure 2.1 An isolation plate showing the central inoculum and the gliding bacteria moving away from the centre. A pea sized quantity of dung was placed in the centre of an MTY agar plate which was incubated at 25°C for 24 hours. Organisms displaying gliding motility that had moved away from the central inoculation were picked off and investigated as possible *Vitreoscilla* strains.

Table 2.1 Isolates of bacteria from various sources of dung. Possible *Vitreoscilla* were identified using to cell dimension, colony morphology, type of movement and growth in chains.

Isolate	Source	Gram Stain	Motile by Gliding ?	Cells in Chains ?	Possible <i>Vitreoscilla</i> ?
LB1	Cow	Neg.	Yes	No	No
LB2	Cow	Pos.	No	No	No
LB3	Cow	Neg.	Yes	No	No
LB4	Cow	Neg.	No	No	No
LB5	Cow	Pos.	No	No	No
LB6	Cow	Neg.	No	No	No
LB7	Cow	Neg.	Yes	Yes	Yes
LB8	Cow	Neg.	Yes	Yes	Yes
LB9	Cow	Neg.	Yes	Yes	Yes
LB10	Fresh cow	Pos.	No	No	No
LB11	Old cow	Neg.	No	No	No
LB12	Old cow	Neg.	Yes	Yes	Yes
LB13	Old cow	Neg.	Yes	Yes	Yes
LB14	Sheep	Neg.	No	Yes	No
LB15	Horse	Neg.	Yes	Yes	Yes
LB16	Horse	Pos.	No	Yes	No
LB17	Cow	Neg.	No	Yes	No
LB18	Cow	Neg.	No	Yes	No

2.3.2 Purification of cultures

Plating techniques were developed to optimise the recovery of gliding bacteria and were initially based on published work (Pringsheim, 1951). After isolation it was necessary to obtain a pure culture; this would usually be achieved by streaking the organism onto a fresh plate. It was not possible to purify the *Vitreoscilla* isolates in this manner since mucous produced by the cells trapped contaminating bacteria and single colonies were not formed. A new method using lysozyme was developed to overcome this problem. The mucus produced by *Vitreoscilla* is made up of polysaccharides (Youderian, 1998). As lysozyme hydrolyses some polysaccharides it was tested for its ability to hydrolyse the polysaccharides in the mucous produced by *Vitreoscilla* and aid purification of the strains isolated.

Cells were treated as described in the Materials and Methods section (Page 38). After repeated trials the purification protocol was successful on two of the isolates (LB13 and LB9) producing colonies which were derived from single trichomes without the contaminating organisms. Attempts at purification of the other isolates were not successful.

2.3.3 Examination of colony morphology and cell width

Characterisation and confirmation that the isolates were *Vitreoscilla* species began with an examination of the colony morphology of pure cultures and measurement of the width of individual cells. *Vitreoscilla* forms distinctive colonies with individual filaments gliding away from the original point of inoculation. Under low magnification (x100) it is possible to see the trail of mucus left behind by the organism. The widths of cells were also measured to give an indication of which species of *Vitreoscilla* had been isolated. The width of individual cells was

measured using a microscope fitted with an eyepiece graticule (Olympus, Tokyo), calibrated with a stage graticule (Pyser, Kent). A selection of 10 cells grown on a slide on MTY agar were measured and an average calculated. The average width of the LB9 cells was found to be $1.33 \mu\text{m} \pm 0.067 \text{ SEM}$ and the average width of LB13 was found to be $1.38 \mu\text{m} \pm 0.041 \text{ SEM}$.

2.3.4 Movement of isolate LB13

A closer examination of the movement of one of the isolates was made. A small quantity of sterile molten MTY agar number was dropped onto a sterile slide and allowed to set. An inoculation of LB13 was made on the agar and the slide was incubated at 25°C in a Petri dish with a damp piece of filter paper. After 24 hours incubation the slide was observed for gliding movement. Observation of LB13 on thin agar slides showed the organism moving across the agar, chains of cells were seen moving in spirals and leaving behind a visible trail.

2.3.5 The effect of sodium chloride concentration on the growth of LB9 and LB13

If the terminal oxidase of *Vitreoscilla* is a sodium ion pump then the organism must require sodium ions for this enzyme to function. It was therefore important to determine at what concentration the isolates required sodium, if at all. It was also desirable to determine the concentration of sodium ions and pH in their natural environment.

A flame photometer (Corning 480, Halstead Essex) was used to determine the concentration of sodium chloride in sample of cow dung. One gram of dry dung was made up to 20 ml in double distilled water. The sample was left at room temperature for 1 hour and was then filtered. The sample was then run through the flame photometer. Indicator paper was used to determine the pH of wet cow

dung. The concentration of sodium ions tested in cow dung was found to be 8 mM this equates to 0.148 % of the wet weight. The pH of the dung was found to be 7.3.

The effect of sodium chloride on the growth of isolates LB9 and LB13 was tested in MTY agar. Agar was prepared with 0, 0.5, 1, and 1.5 % added sodium chloride. The individual ingredients of the agar and broth also contained a small quantity of sodium. This means that the actual concentration was slightly higher than the percentage stated (Table 2.2 Page 46). The isolates were grown for 48 hours on MTY agar; approximately 0.1 grams of each organism was then added to 1 ml of 170 mM potassium chloride solution. The samples were mixed and diluted to 10^{-5} , 10^{-6} , and 10^{-7} with 170 mM potassium chloride solution. Each dilution (0.1 ml) was spread onto three replicate plates at each sodium chloride concentration. The plates were incubated at 25°C for 24 hours and the colonies were counted. *Vitreoscilla* colonies had to be counted after only 24 hours before the colonies had merged. After 48 hours the plates were re-examined as there appeared to be a variation in the colony sizes on the different plates and so at this point colony size was measured.

Table 2.2 Total concentrations of sodium chloride in the agar and broth, calculated from the content of sodium chloride in the individual ingredients.

Sodium chloride added (g l ⁻¹)	Total sodium chloride concentration (g l ⁻¹)	
	Agar	Broth
10	10.8	10.7
7.5	8.3	8.2
5	5.8	5.7
2.5	3.3	3.2
0	0.8	0.7

Figure 2.2 (Page 48) shows the colony counts for LB9 and LB13 versus sodium chloride concentration. LB9 grew well at concentrations up to and including 1% added sodium chloride. Above 1% added sodium chloride the number of colony forming units started to decline. LB13 grew well at 0% added sodium chloride. The general trend was down, at 1.5% added sodium chloride no growth was detected for either organism at the lowest dilution plated (10^{-5}).

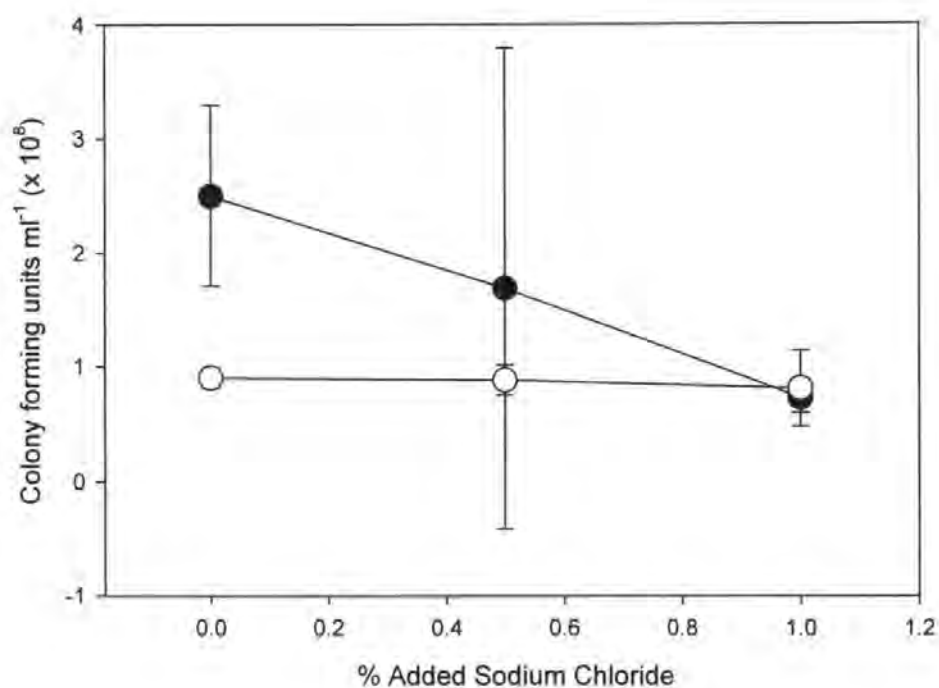


Figure 2.2 The effect of sodium chloride concentration on the growth of isolate LB9 \circ and LB13 \bullet . Cultures were grown on agar containing 0, 0.5, 1, 1.5 % added sodium chloride at 25°C for 24 hours. The average of three replicates is shown \pm 1 standard deviation. At 1.5 % added sodium chloride no colonies of either organism were present giving a count of $< 10^6$.

Figures 2.3 and 2.4 (Page 50) show a representative plate from each sodium chloride concentration; the differences in colony morphology at the different concentrations can clearly be seen. Figure 2.5 (Page 51) shows the effect of sodium chloride concentration on colony diameter for the two *Vitreoscilla* strains. When no extra sodium chloride was added to the media both isolates had colony sizes of between 3 and 4.5 mm. With 0.5% added sodium chloride LB13 had colonies of between 5 and 7.5 mm whilst LB9 colonies varied between 2 and 4.5 mm. At 1% added sodium chloride both isolates had similar sized colonies of between 1 and 2 mm.



Figure 2.3 Plates showing LB9 growth at three sodium chloride concentrations, 0, 0.5 and 1% added sodium chloride.



Figure 2.4 Plates showing LB13 growth at three sodium chloride concentrations, 0, 0.5 and 1% added sodium chloride.

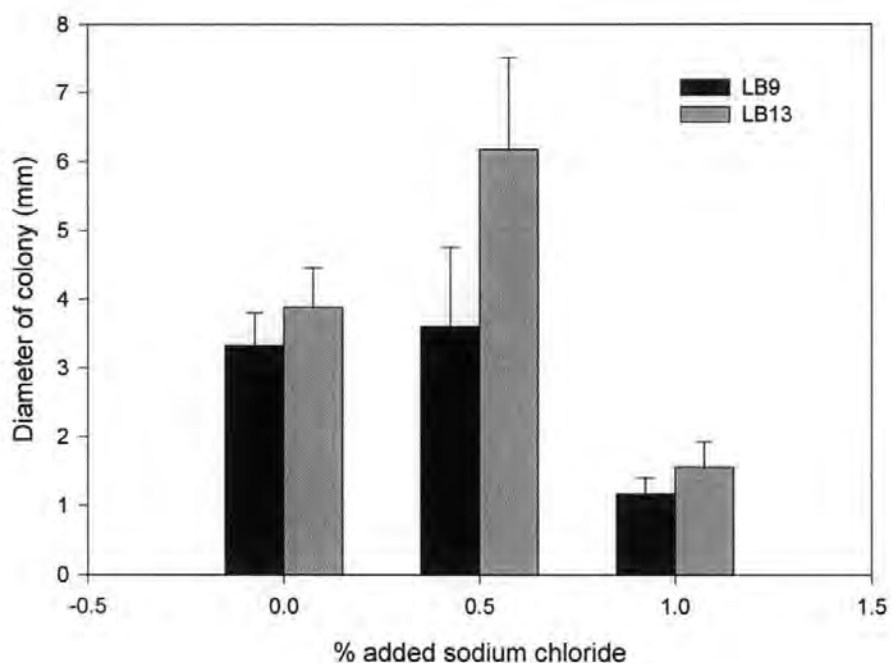


Figure 2.5 The size of *Vitreoscilla* colonies on agar containing 0, 0.5, and 1 % added sodium chloride. The plates were incubated at 25° C for 48 hours. Each bar represents the average of 9 colonies chosen at random measured at the widest point plus or minus one standard error.

The effect of sodium chloride concentration on the growth of LB13 in MTY broth was also tested. Broths were made with 0, 0.25, 0.75, and 1 % added sodium chloride (see Table 2.2 Page 46 for total concentration). One ml of overnight culture was added to three replicate 50 ml batches of broth at each sodium chloride concentration. These broths were then incubated at 25° C in a shaking water bath at 50 rpm. Optical density was measured at 590 nm at 17 hours, 48 hours and 51 hours.

Figure 2.6 (Page 53) shows the optical density of the cultures after inoculation. At all times measured the optimum sodium chloride percentage was 0.25 % with this culture reaching an optical density twice as high as the next highest (0.5 %). Either side of 0.25 % the optical densities of the cultures were lower with 1 % added sodium chloride showing the lowest density.

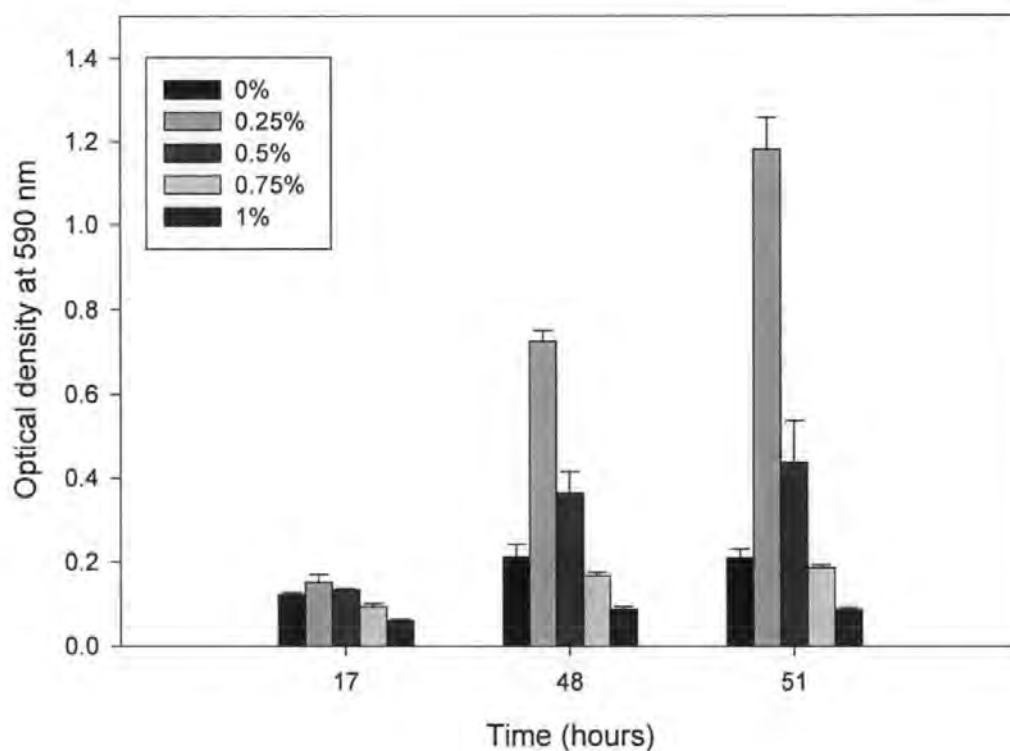


Figure 2.6 The effect of sodium concentration on the growth of isolate LB13 in broth. LB13 was grown in broth containing 0, 0.25, 0.5, 0.75, and 1% added sodium chloride. The flasks were incubated at 25° C in a shaking water bath at 50 rpm. The optical density was measured at 17, 48, and 51 hours.

2.3.6 The effect of FCCP and monensin on the growth of isolates LB13

The effects of monensin and FCCP on the organism were tested. Monensin is a Na^+ specific ionophore that catalyses Na^+/H^+ exchange across membranes (Sandeaux, Sandeaux et al., 1982) and FCCP is an H^+ -specific protonophore (Benz and Mclaughlin, 1983). Monensin should cause the collapse of a Na^+ gradient that was created by a primary sodium pump (Skulachev, 1987). Whereas an organism utilising a primary sodium pump would be only transiently affected by a H^+ -specific protonophore (Efioke and Webster, 1992).

Isolate LB13 was grown on MTY agar containing 10 μM FCCP (Sigma), 10 μM monensin (Sigma, sodium salt) and both 10 μM FCCP and 10 μM monensin. FCCP and/or monensin were filter sterilised and added just before the agar was poured. An overnight culture of LB13 was diluted to 10^{-3} , 10^{-4} and 10^{-5} ; 0.1 ml of each dilution was spread onto three replicate plates of each test agar. The plates were incubated at 25° C for 24 hours and then the colonies were counted. Figure 2.7 (Page 55) shows the colony counts for LB13 grown on agar containing FCCP and/or monensin. There was no difference between the growth of LB13 on agar containing monensin and/or FCCP, and the control.

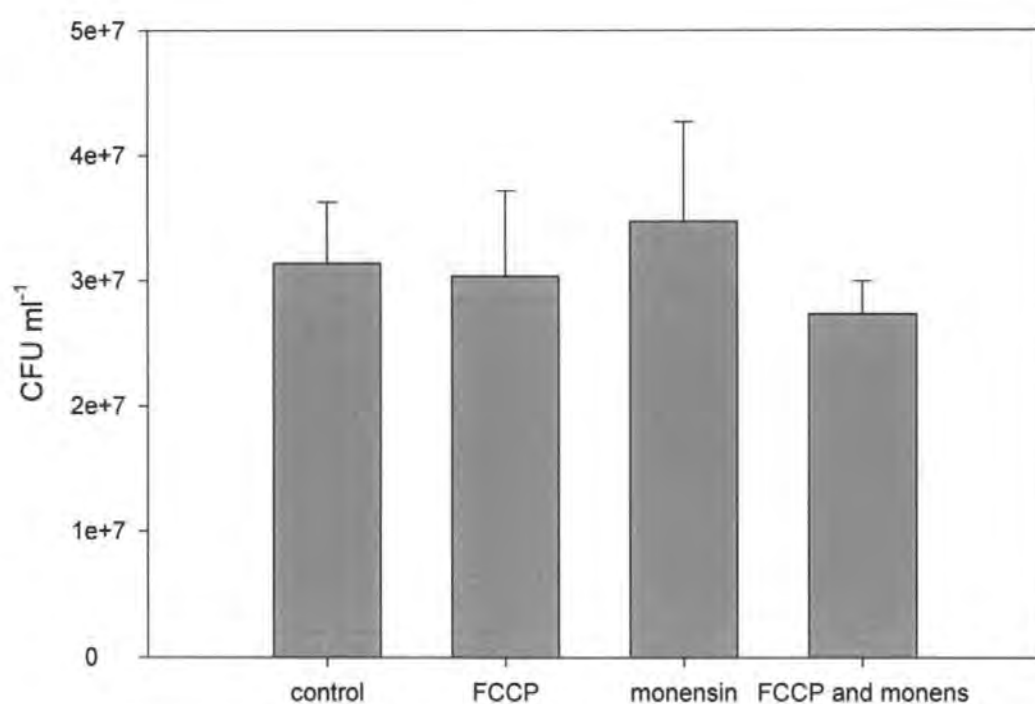


Figure 2.7 The effect of FCCP and monensin on the growth of LB13. Each bar represents Colony forming units plus or minus 1 standard error. LB13 was grown on agar containing 10 μ M FCCP or 10 μ M monensin or 10 μ M of both monensin and FCCP dissolved in ethanol, control plates contained ethanol. The plates were incubated at 25° C for 24 hours.

LB13 was also grown in broth containing 10 μ M FCCP, 10 μ M monensin and both 10 μ M FCCP and 10 μ M monensin to determine whether they would have an effect of growth of the organism in a liquid medium. Overnight culture of LB13 (1 ml) was added to three replicates of each 50 ml test broth and incubated at 25°C in a shaking water bath at 50 rpm. At 17, 24, 41, and 46 hours the optical density of each broth was measured at 590 nm. Figure 2.8 (Page 57) shows the optical density of the broths at 17, 24 and 41 hours after inoculation. After 41 hours there was a slight reduction in the growth of LB13 in all three test broths compared to the control. Table 2.3 (Page 58) shows the P values from the analysis of variance carried out on each pair of results. The critical value is 0.05 at 95 % confidence. At both 17 hours and 24 hours both broths containing FCCP are significantly different from the control whilst the monensin broths are not. At 41 hours none of the results are significantly different.

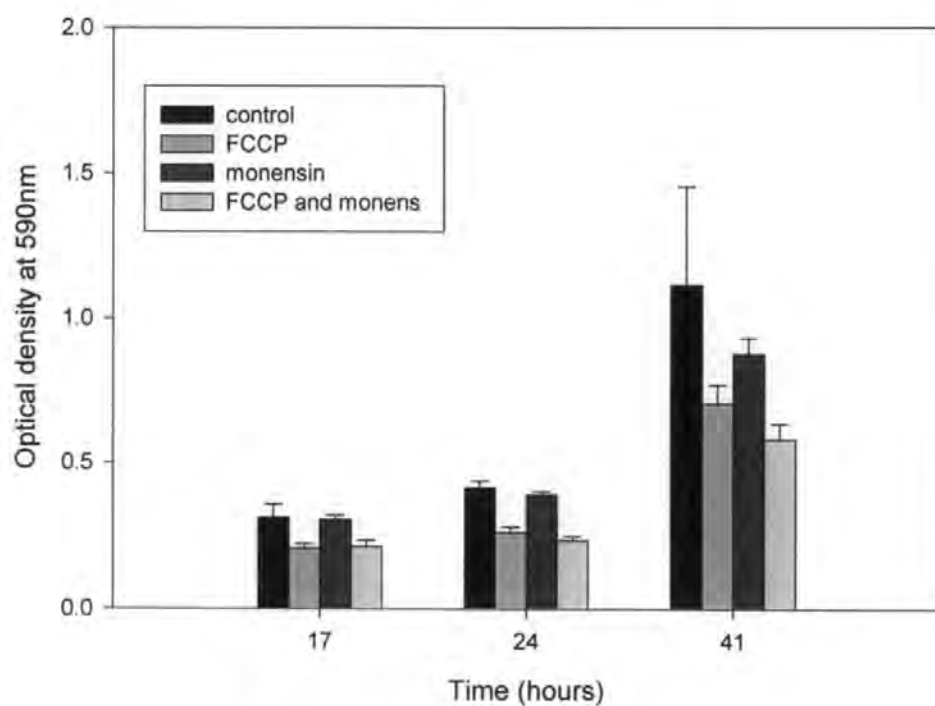


Figure 2.8 The effect of FCCP and/or monensin on the growth of LB13 in broth. LB13 was grown in broth containing 10 μ M FCCP and/or 10 μ M monensin. The broths were incubated at 25° C in a shaking water bath at 50 rpm. At 17, 24 and 41 hours the optical densities of the broths were measured.

Table 2.3 P values for the analysis of variance between the absorbance at 590 nm of control and experimental broths after 17, 24 and 41 hours of growth with FCCP and/or monensin. At 95 % confidence, critical value is 0.05. *indicates results significantly different from the control.

Treatment	P values		
	17 hours	24 hours	41 hours
FCCP	0.0357*	0.0021*	0.1665
FCCP and Monensin	0.0487*	0.0008*	0.0918
Monensin	0.8379	0.2736	0.3782

2.3.7 The effect of potassium chloride on the growth of isolate LB13

It has been reported that moderate concentrations of potassium ions are lethal to *Vitreoscilla* (Efiok and Webster, 1990) and so the effect of potassium chloride concentration on growth was determined. Isolate LB13 was grown in broth containing 5, 10, 25, 50, and 100 mM potassium chloride. One ml of overnight culture was inoculated into each of three replicate 50 ml broths at each potassium chloride concentration. The flasks were incubated at 25°C in a shaking water bath at 50rpm. At 24 and 48 hours the optical density at 590 nm of each broth was measured. No difference was observed between the control and test broths after 24 hours.

2.4 Discussion

2.4.1 Isolation

The absence of *Vitreoscilla* in the excrement of other grazing animals may be due to variation in the pH of the dung (although only the pH of cow dung was tested). It may also be the case that nutritional requirements that are met by the cow dung are absent in the other dungs. Most likely, however, it is because cow dung has a different consistency than sheep or horse dung. A crust is often formed over the surface whilst the interior remains moist; this provides a wet environment long after sheep and horse dung has become desiccated.

Three trips were made to the moor to collect samples. Six isolates were recovered during the first visit on the 25/2/98; none of these isolates proved to be *Vitreoscilla*. The second visit took place on the 21/8/98 and yielded 10 possible *Vitreoscilla* of which 6 were confirmed. The final visit was on the 16/10/98 and two isolates were retained, neither of which proved to be *Vitreoscilla*. The apparent seasonality of the presence of *Vitreoscilla* could be due to temperature. The sampling site is approximately 200 meters above sea level (grid reference 5555 6465) and experiences on average a temperature 2 °C below that at sea level. The absence of *Vitreoscilla* in the winter samples raises the question, where are the organisms in the winter ? *Vitreoscilla* is not known to produce a resting stage and so it is unlikely that they over winter as spores. Further investigation would be needed to determine why the organism is not culturable from dung samples in the colder months.

The method of isolation proved effective, yielding a number of *Vitreoscilla* on which to carry out further work but improvements could have been made. The

mode of movement of the *Vitreoscilla* is such that they require a surface to glide on. As a result, when fungi are present the *Vitreoscilla* will use the fungal hyphae as a substrate on which to glide, making isolating them from the agar impossible. It would be possible to add antifungal agents such as amphotericin B or an azole based antifungal to the agar to reduce the fungal growth. Amphotericin B is a polyene antibiotic that binds to membrane sterols and affects the permeability of the fungal membrane (Ghannoum and Rice, 1999). Prokaryote membranes do not contain sterols and so polyene antibiotics do not affect them, it is therefore unlikely that *Vitreoscilla* would be affected by the use of this antibiotic. Azole based antibiotics such as fluconazole block the production of ergosterol resulting in a plasma membrane with altered structure and function. Unlike polyene antibiotics azole based antibiotics do affect some bacteria and so experimentation would be necessary (Ghannoum and Rice, 1999).

On many plates Gram positive bacteria such as *Bacillus* species overran the plates and any *Vitreoscilla* growing on them. This problem could possibly be solved by the addition of gram positive specific antibiotics such as the macrolides erythromycin or clarithromycin. As with antifungals the effect of this addition on *Vitreoscilla* would need to be investigated. The macrolide antibiotics prevent protein synthesis and are mainly effective against gram positive bacteria although some gram negative organisms are susceptible (Russell, 1998).

2.4.2 Purification

Difficulties were experienced in purifying environmental isolates. Repeated streaking failed to produce a pure culture. Contaminating bacteria were found to adhere to the mucous layer of the *Vitreoscilla* and were carried with them during streaking. It was therefore necessary to develop a new method. Lysozyme was

tested to determine whether it would break down the mucous layer that surrounds the bacteria and traps contaminating bacteria. The mucous produced by *Vitreoscilla* is made up of polysaccharides (Youderian, 1998), which can be broken down by the addition of lysozyme. Single colonies were achieved for 2 of the isolates but not the other 4. As only one isolate was required for the study no further time was spent refining the procedure. A higher concentration of lysozyme or a longer exposure time may have proved successful in purifying the remaining cultures but a balance would need to be struck between obtaining single colonies and lysing the organism.

2.4.3 Problems with the enumeration of *Vitreoscilla* cultures

During the initial characterisation of the *Vitreoscilla* isolates it became apparent that problems would occur in quantifying the cell density. Broth cultures can be quantified in one of two ways. The culture can be diluted and spread on to agar for a direct count or the optical density of the culture can be measured to give a comparison with other cultures. Both these methods proved problematic with *Vitreoscilla* because of its mode of growth. The formation of trichomes and associated mucus means that above a certain density the bacteria begin to flocculate in broth culture giving an artificially low and erratic optical density reading. Plate counts are also prone to error for the same reason with one colony forming unit arising from a highly variable number of cells in a trichome. Plate counts are also difficult because of the gliding motility of the organism, which means that the colonies overrun each other. They must be counted when the colonies are still very small and individual at which point they are difficult to see.

To a certain extent these problems were overcome. The problem of flocculation can be reduced with vigorous shaking of the culture prior to sampling. This,

however, slows the growth of the organism and can therefore not be used on ongoing cultures. Sampling of broth cultures must be done in a way to minimise the effects of removing a sample with a large "lump" of cells in it and thus giving an artificially high count. The only sure way of eliminating this form of inaccuracy is to stop broth cultures before the point at which they begin to flocculate. In order to reduce problems with plate counts it is also necessary to employ vigorous shaking when performing the dilution steps.

Despite these precautions it must be noted that plate counts and an optical density readings may give artificially low results, with a tendency to become more variable at high cell densities.

2.4.4 Identification

Group 15 of Bergey's Determinative bacteriology (Holt, Krieg, Sneath, Stanley, and Williams, 1994) are the nonphotosynthetic, nonfruiting gliding bacteria. Of these only the *genus Vitreoscilla* contains bacteria that are rod shaped, form multicellular filaments not enclosed in a sheath and do not produce pigments (Holt, Krieg, Sneath, Stanley, and Williams, 1994). Both LB9 and LB13 fit this description and were therefore identified as species of *Vitreoscilla*.

Cell width along with habitat and growth on nutrient media are distinguishing features of the three *Vitreoscilla* species that are currently recognised (see Chapter one Page 2). Only *Vitreoscilla stercoraria* has been isolated from cow dung and will grow on nutrient agar. The other two species originate from fresh water habitats. *Vitreoscilla stercoraria* can also be distinguished from *Vitreoscilla beggiatoides* and *Vitreoscilla filiformis* by the width of its cells and by trichome morphology (Strohl, Schmidt et al., 1986). *V. stercoraria* cells measure 1 μm in

diameter while *V. filiformis* cells measure between 1 and 1.5 μm . *V. beggiatoides* has the largest cell width with cells measuring 2.5 to 3 μm in diameter (Holt, Krieg, Sneath, Stanley, and Williams, 1994). Isolates LB9 and LB13 had cells with a width of 1.33 μm and 1.38 μm respectively, were both isolated from cow dung and grew on nutrient agar. The cell widths were slightly larger than those published for *V. stercoraria*, however the other observations make it probable that both isolates can be identified as *V. stercoraria*.

2.4.5 The effect of sodium chloride on growth

In order to determine whether the two isolates had a requirement for sodium chloride the organisms were grown in media containing different concentrations. Both grew well on agar containing no added sodium chloride; this medium did however contain a small amount of sodium in its constituents. Above 1 % added sodium chloride LB9 numbers started to drop and above 0.5 % LB13 began to decline in numbers. A requirement for sodium chloride was not proved because it could not be completely removed from the media but both organisms showed some ability to tolerate added sodium chloride in agar. None of the previous studies of *Vitreoscilla*'s terminal oxidase or nutritional requirements looked at the requirement of sodium for growth.

An interesting effect of the sodium chloride on the gliding of the organisms was noted. The bacteria growing on the no added sodium chloride plates produced colonies that did not spread (Figures 2.3 and 2.4 Page 50). This could be explained by the amount of moisture available on the plate, at the time the experiment was conducted the effect of moisture on the gliding behaviour of the organism was not known. On very dry plates *Vitreoscilla* colonies tend to be smaller with less gliding taking place. This observation was tested by a student

and it was not found possible to recreate the previous results. This is probably because the wetness of the plate is important in determining how much *Vitreoscilla* will glide. It is possible that the differences in surface moisture levels on the original plates is responsible for the differences in colony morphology observed.

Isolate LB13 was tested further in broth because initial cultures showed that it grew more successfully in liquid culture than LB9. Isolate LB13 showed a requirement for sodium chloride in liquid culture with an optimum of 0.25 %. The broth culture containing 0.25 % added sodium chloride produced twice the density of the no added sodium chloride broth

2.4.6 The effect of FCCP and monensin on growth

Neither FCCP nor monensin affected the growth of LB13 on agar (Figure. 2.7 Page 55). However, FCCP, but not monensin inhibited growth of LB13 in liquid culture (Figure. 2.8 Page 57). This suggests that the organism is not using a primary sodium pump. It is predicted that FCCP would have no effect on an organism using a pump while monensin is predicted to inhibit.

At 17 hours and 24 hours the FCCP did have a significant effect on the growth of LB13 and monensin did not but at 41 hours the effect of variation in the control made the result statistically insignificant.

2.4.7 The effect of potassium on growth

Potassium did not have an effect on the growth of isolate LB13. Efiok and Webster quoted unpublished results in their 1990 paper (Efiok and Webster, 1990) claiming that *Vitreoscilla* is extremely sensitive to moderate concentrations of

potassium. In later papers this is not mentioned and no evidence is produced to support this claim.

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Chapter 3

Purification of cytochrome *bo* from *Vitreoscilla* strains LB13 and C1

3.1 Introduction

The first step in the purification of a membrane protein is to release the membrane from the cell. This can be achieved in a number of ways depending on the type of cell and the amount of force required to break it open, the cells can be homogenised, passed through a French press, placed in a blender, or subjected to ultrasound. Hypo-osmotic lysis can be used if the cell walls are not particularly robust but for stronger cells such as gram positive bacteria, lysozyme will need to be added to aid disruption. Once released from normal cellular environments proteins must be protected from denaturation. The pH and osmolarity of the media must be controlled, all manipulations must be carried out on ice and protease inhibitors must be added, for example Ca^{2+} chelators to inhibit calcium dependent proteases, PMSF (phenylmethylsulfonyl fluoride) a serine protease inhibitor, and leupeptin, a thiol protease inhibitor. Once the membranes have been released (and isolated from other cell debris using centrifugation) the protein can be removed from the membrane using detergent, for example: Triton X-100 or deoxycholate (Findlay, 1990).

Once the protein has been solubilised further purification can be achieved using column chromatography. Ion exchange, reverse phase/hydrophobic interaction or gel filtration can be used to achieve an increase in the purity of the protein. A final polish of the purification can be achieved using gel filtration or affinity chromatography.

Isolation and purification of cytochrome *bo* from *Vitreoscilla* has been described by Georgiou and Webster (Georgiou and Webster, 1987). First, spheroplasts of the membranes were isolated using buffer containing lysozyme and sucrose. The recovered spheroplasts were frozen overnight and then resuspended in buffer

containing DNAase I and II as well as RNAase A. The recovered membranes were then solubilised using a complicated bile salt membrane fractionation method. A series of dialysis steps were then employed before the enzyme was concentrated using ultrafiltration. A Sephadex G-200 superfine column was the final step in the purification procedure.

As a French press was not available a method to isolate cytochrome *bo* from *Vitreoscilla* was devised based upon the method that Moody and Rich had used to isolate *bo* from *E. coli*. Most papers dealing with Purified cytochrome *bo* from *E. coli* follow the protocol of Matsushita *et al* (Matusuhita, Patel et al., 1986). In this procedure the cells are grown to log phase then harvested by centrifugation. The cells were then passed through a French press and PMSF and leupeptin were added. This procedure was altered slightly by Moody *et al* who added DNAase to the cells prior to passing them through the French press (Moody, Rumbley et al., 1993) and by Moody and Rich who replaced the use of a French press with a Bead Beater to disrupt the cells (Moody and Rich, 1994). In all cases the membranes were recovered using centrifugation.

Purification of the enzyme is also detailed by Matsushita *et al* and starts with treatment of the membranes with urea and cholate. The enzyme is then extracted from the membrane using octylglucoside. The extracted enzyme is passed down a DEAE Sepharose CL-6B column followed by a ω -aminohexyl agarose column. The purified enzyme is then concentrated by ultrafiltration.

Growth conditions for the isolated strain of *Vitreoscilla* were optimised for sodium chloride concentration and aeration.

RG145 is a strain of *E coli* that over produces cytochrome *bo* five fold and does not produce cytochrome *bd* (Au and Gennis, 1987). RG145 was used to develop the method for the isolation of respiratory membranes with the intention of comparing cytochrome *bo* from RG145 with that of *Vitreoscilla*. The initial protocol for the isolation and purification of cytochrome *bo* from RG145 came from a published method (Cheesman, Watmough et al., 1993;Moody and Rich, 1994). This protocol was developed for use on *Vitreoscilla*.

The membranes were washed prior to extraction with urea and cholate, without this step the enzyme failed to adhere to the DEAE column. In order to determine whether the urea and cholate denatured the *bo*, membranes were incubated in urea and/or cholate at the concentration used for the wash and the amount of *bo* was monitored spectrophotometrically.

Purification of the extracted enzyme began with ion exchange chromatography on a DEAE-Sepharose CL-6B column. The enzyme was purified further using a hydrophobic interaction column.

3.2 Materials and methods

Centrifugation steps were carried out in an MSE Europa 24 (MSE Scientific Instruments, Crawley, UK) unless otherwise stated. All spectrophotometer readings were measured on a Perkin Elmer spectrophotometer (Perkin Elmer Lambda Bio 20 dual beam, Wellesley, Massachusetts). All measurements made of cytochrome *bo* using the extinction coefficient $20.5\text{mM}^{-1}\text{cm}^{-1}\Delta_{560-580\text{nm}}$ also include cytochrome *bd*. Protein measurements were made using a commercially available kit according to the manufacturer's instructions (Kit number 690-A, Sigma, Poole, Dorset). The membranes and purified enzyme were stored at -80°C .

3.2.1 Overview of the purification process

Figure 3.1 (Page 73) shows a flow diagram of the purification process starting with the scale up of growth of *Vitreoscilla*. The diagram will be referred to in the materials and methods section as each step is detailed.

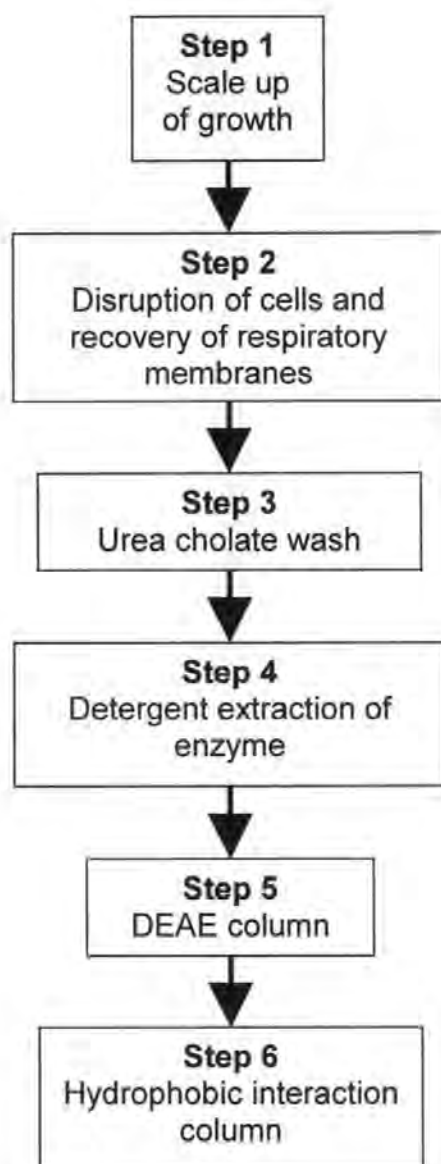


Figure 3.1 Flow diagram of the six main steps in the purification of cytochrome *bo* from *Vitreoscilla stercoraria*. The detail of each step is given in the Materials and Methods section.

3.2.2 Optimisation of growth conditions and scale up of growth

The optimum NaCl concentration for growth of *Vitreoscilla* LB13 was determined during the initial characterisation of the *Vitreoscilla* isolates (see Chapter 2 Page 40).

Step 1 (see Figure 3.1 Page 73) in the purification of cytochrome *bo* from *Vitreoscilla* was the scale up of growth from small 250 ml flasks holding 50 ml of broth to 2000 ml flasks containing 700 ml of broth. Aeration conditions were tested by growing the organism in 2 litre flasks containing 700 ml of MTY broth (see Chapter 2 Page 39) and varying the aeration conditions between the flasks. Air that was pumped into the flasks was passed through Whatman Hepa-Vents (pore size 0.3 μm ; supplied by Merck Eurolab Ltd, Poole, UK) before entering the culture vessel. The silicone bungs had an air inlet and an air outlet. The flasks were incubated for 48 hours at 25 °C and the cells were then harvested by centrifugation at 9000 g_{av} and the pellet weights measured.

After analysis of the results from the aeration experiment the final method for growth of *Vitreoscilla* was as follows. The organism was streaked from liquid nitrogen storage onto MTY agar (see Chapter 2 Page 39) and incubated at 25 °C for 48 hours. A colony of the bacteria was removed from the plate with a loop and transferred into a 250 ml conical flask containing 50 ml of MTY broth. The flask was incubated at 25 °C for 48 hours. Pre culture (10 ml) was then inoculated into 6, 2 litre flasks containing 700 ml of MTY broth. The flasks were incubated at 25 °C in a model G25 orbital shaking incubator (New Brunswick Scientific, Edison, New Jersey) at 50 rpm for 48 hours. Air was pumped into the flasks through a Hepa-vent directly into the broth at a rate of 200 ml min⁻¹. Waste air was expelled from the flasks through Hepa-vents. Cells were harvested using by centrifugation

at 9000 g_{av} and were washed twice in buffer A (see Appendix A Page 181). The washed cells were resuspended in buffer A and stored at $-80\text{ }^{\circ}\text{C}$ until needed.

3.2.3 Isolation of membranes: method development with *Escherichia coli* RG145

Step 2 in the purification process (see Figure 3.1 Page 73) was the disruption of the cells followed by recovery of the respiratory membranes. For reasons detailed in the introduction *Escherichia coli* RG145 was used to develop this procedure. *Escherichia coli* RG145 was grown in 2 litre baffled flasks in 700 ml of broth containing 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 6.8 g l^{-1} KH_2PO_4 , 10 g l^{-1} and glycerol (BDH) 270 μM ampicillin, 10 μM CuSO_4 , 200 $\mu\text{l l}^{-1}$ antifoam (BDH) adjusted to pH 7.4 with 30 % NaOH. After a chance observation that it increased yield 0.25 g l^{-1} MgSO_4 was added to the medium. The flasks were incubated at $37\text{ }^{\circ}\text{C}$ for 16 hours, in a New Brunswick scientific orbital shaking incubator model G25 at 300 rpm and air was pumped into each flask through a Hepa-vent at a rate of 200 ml min^{-1} .

RG145 (50-100g wet weight) was added to the chamber of a Bead Beater (75 ml; Biospec Products, Bartlesville, OK). Glass beads (80 g of 1 mm diameter, BDH, Poole, UK) were added and the remaining volume was filled with buffer B (see Appendix A Page 181). The Bead Beater was turned on for 20x15 second bursts with 45 seconds cooling time allowed between bursts and PMSF (0.2 ml of 200 mM), 0.2 ml of 2 mM leupeptin and 0.2 ml of 2 mM pepstatin were added to the chamber. The mixture was centrifuged at 3000 g_{av} for 10 min to remove any unbroken cells. The supernatant was centrifuged for 30 min at 35 000 g_{av} . The pellet was resuspended in buffer B and made up to 10 ml. A difference spectrum was measured and the quantity of cytochrome *bo* was determined using an extinction coefficient of $20.5\text{ mM}^{-1}\text{cm}^{-1}\Delta_{560-580\text{nm}}$.

Once the yield was sufficiently high the protocol was adapted for *Vitreoscilla*. The final protocol was as follows. Stored *Vitreoscilla* cells (30-50 g) were added to the bead beater chamber together with 0.2 g lysozyme (Sigma L6876 from chicken egg white), 0.05 g DNAase I (Sigma DN25 from bovine pancreas), MgSO_4 (200 μl of 10 mM), and 80 g of glass beads (1 mm diameter). The remaining volume was filled with buffer A. The chamber was incubated at 25°C for 15 min. The cells were disrupted with 20x15 second bursts with 45 s of cooling time between bursts.

Half a tablet of Complete inhibitor (Roche Molecular Bioproducts, 'Complete' tablets contain serine, cysteine and metallo- protease inhibitors) was added to the chamber. The mixture was centrifuged at 3000 g_{av} for 10 min to remove any unbroken cells. The supernatant was centrifuged for 30 min at 35000 g_{av} for 30 min. The pellet was resuspended in buffer B and made up to 10 ml. A difference spectrum was measured and the quantity of cytochrome *bo* (and *bd*) was determined using the extinction coefficient $20.5\text{mM}^{-1}\text{cm}^{-1} \Delta_{560-580\text{nm}}$ (Moody and Rich, 1994).

3.2.4 Urea cholate wash

The urea cholate wash was step 3 in the purification procedure (see Figure 3.1 Page 73). Stored membranes were thawed and diluted to 50 ml in buffer B. Urea (50 ml of 10 M) was added. The mixture was then centrifuged at 35000 g_{av} for 2 hours in a Beckmann L755 ultracentrifuge and the pellet resuspended to 45 ml with buffer B. 20 ml of 20% sodium cholate was added. The mixture was centrifuged at 35000 g_{av} for 2 hours and the pellet was resuspended up to 80 ml in buffer B. The mixture was then centrifuged again at 35000 g_{av} for 2 hours and the pellet was resuspended up to 10 ml in buffer C (see Appendix A Page 181).

3.2.5 Detergent extraction of the cytochrome *bo*

Step 4 of the purification was the extraction of cytochrome *bo* from the membrane using octylglucoside. The octylglucoside concentration was tested at 0.15 %, 0.25 %, 0.5 %, 1.0 %, 1.5 %, and 2.0 %. All concentrations were tested in duplicate. The detergent was added to 800 μ l of double distilled water in a 2000 μ l Eppendorf tube and 200 μ l of membranes were added. The tubes were incubated at 4°C for 30 mins. The tubes were then centrifuged in a microcentrifuge (Sanyo Gallenkamp PLC, Loughborough Leics) at 13000 g_{av} for 15 mins. The size of the pellet was measured using a ruler. A difference spectrum of the supernatant was measured on a Perkin Elmer spectrophotometer and the quantity of cytochrome *bo* was determined.

A second trial was conducted using the protein concentration of the membrane sample to determine the detergent concentration. The protein content of the membrane solution was measured. Detergent to protein ratios of 1:1, 1:4, 1:6, 1:8, 1:9, and 1:10 were tested. All concentrations were tested in triplicate. The detergent was added to 600 μ l of double distilled water in a 2000 μ l Eppendorf tube; 400 μ l of thawed membranes were added. The tubes were incubated for 30 min at 4°C and then centrifuged at 13000 g_{av} for 15 min. The pellets were measured using a ruler, difference spectra of the supernatants were measured and the quantity of cytochrome *bo* was determined.

After analysis of the results the final method for the extraction of the enzyme was as follows; octylglucoside was added to the membrane solution at a ratio of 0.75 % detergent to protein. This was mixed and allowed to incubate at 4°C for 30 min.

The mixture was then centrifuged at 13000 g_{av} for 15 min. and the supernatant retained. A difference spectrum of the supernatant was measured and the quantity of cytochrome *bo* was determined.

3.2.6 Inhibition of extracted cytochrome *bo* by KCN

Extracted enzyme (1.5 μ l of 1.9 μ M), 1 μ l of purified enzyme (3 μ M) or 1 μ l of membranes (2.3 μ M) was incubated in 250 μ l of buffer A with either 1 μ l of 100 mM KCN or 1 μ l of water. The reaction was started by the addition of 1 μ l of 2.6 mM decylubiquinol. The change in absorbance at 262 nm was monitored. An exponential rise to maximum was fitted to the data and the initial rate was used to determine inhibition level.

3.2.7 Preparation of DEAE Sepharose column CL-6B column

Step 5 (see Figure 3.1 Page 73) in the purification was the DEAE column. The DEAE Sepharose column 1.5 cm x 30 cm (ID x length) bed volume 53 ml (Sigma, Poole Dorset) was equilibrated with 3 column volumes of buffer C. The sample was applied at a rate of 15 mlhr⁻¹ and the column was washed with one column volume of buffer C at the same flow rate. The cytochrome was eluted with a 150 ml linear gradient (50-350 mM potassium phosphate) at a flow rate of 15 mlhr⁻¹. Fractions were collected every 8 min (2 ml).

All fractions with an absorbance at 407nm of 0.009 or greater were pooled and concentrated to 2 ml in a Centricon concentrator (Amicon, Inc., Beverly, MA). The pooled fractions were dialysed overnight against 3 changes of buffer E (see Appendix A Page 181). The resulting sample was stored in liquid nitrogen.

3.2.8 Hydrophobic interaction column ω -amino-hexyl-agarose.

Step 6 (see Figure 3.1 Page 73) was the final step in the purification and used a hydrophobic interaction column. The ω -amino-hexyl-agarose column 1.5 cm x 10 cm (ID x length) bed volume 18 ml ml (Sigma, Poole Dorset), was equilibrated with 3 column volumes of buffer E. The sample was applied at a rate of 15 mlhr⁻¹. The sample was eluted with a 60 ml linear gradient (0-250mM K₂SO₄) at a flow rate of 15 mlhr⁻¹. All fractions with an absorbance at 407nm of 0.05 or greater were pooled and concentrated to 2 ml in a Centricon concentrator. The sample was split into 500 μ l aliquots and stored in liquid nitrogen.

3.3 Results and discussion

3.3.1 Optimisation of growth conditions and scale up of growth

Vitreoscilla produce a bacterial haemoglobin; the function of this haemoglobin is not fully understood but it would seem to play a role in enabling the organism to tolerate low oxygen environments as the production of haemoglobin is increased when the organism is grown under low oxygen conditions (Boerman and Webster, 1982; Lamba and Webster, 1980). *Vitreoscilla* also produce cytochrome *bd* and under low oxygen conditions the production of *bd* is also increased (Georgiou and Webster, 1987). It was important to provide sufficient oxygen during growth of the organism to limit the production of these components of the respiratory chain. *Vitreoscilla* prefers to grow on a solid surface. The organism will grow to a higher density if allowed to adhere to the inside surface of the flask in which it is growing. This means that shaking vigorously is not a good option for aeration of the culture; it prevents the bacteria from forming clumps in liquid media and from growing on the surface of the vessel. The first step in the purification of cytochrome *bo* (see Figure 3.1 Page 73) from *Vitreoscilla* was to optimise the large scale growth of the organism. Various aeration options were therefore explored (see Table 3.1 Page 81).

Table 3.1 Aeration conditions of flasks containing *Vitreoscilla stercoraria* strain LB13, for details see Materials and Methods section Page 74

Flask	Conditions	Yield gl ⁻¹
1	Still with cotton wool in neck	1.03
2	Shaking with cotton wool in neck	2.63
3	Still with air pumped into flask through bung	5.56
4	Shaking with air pumped into flask through bung	5.47
5	Still with air pumped into broth through bung	7.32

The flask that was not shaken and had a bung made from non-absorbent cotton wool produced the lowest yield (1.03 g l^{-1}). The flask that was not shaken and had air pumped directly into the broth through a silicone bung produced the highest yield (7.32 g l^{-1}).

Although the highest yield was produced by a flask that was not shaken it was decided that some movement of the flasks was desirable to prevent the production of cytochrome *bd* and also bacterial haemoglobin. It was for this reason that the final chosen protocol for the growth of *Vitreoscilla* includes shaking the culture at 50 rpm.

Wet weight was used to quantify the yield of each growth condition. The weight could be affected by mucus produced by the organism. It is possible that under different growth conditions, different amounts of mucus are produced, complicating the interpretation of results. Other methods of enumeration are also problematic for *Vitreoscilla*; wet weight was the quickest and most straightforward method available.

3.3.2 Isolation of respiratory membranes

Once optimum growth conditions had been established the next step in the purification was to isolate the respiratory membranes from *Vitreoscilla* (see Figure 3.1 Page 73). In order to develop the protocol for the isolation of cytochrome *bo* *Escherichia coli* RG145 was used. This organism is not only easier to grow than *Vitreoscilla* but over produces cytochrome *bo* five fold. RG145 also does not produce cytochrome *bd* making it easier to develop the isolation and purification method. The first trial in which simple disruption of the cells in a Bead Beater was tried (see Materials and Methods section Page 75) produced very little respiratory

membranes that were impossible to quantify because of light scattering and so modifications were made. The protocol was rerun and the slightly coloured supernatant from the centrifugation step was recentrifuged at 35000 g_{av} for two hours. This would determine whether the membranes had fragmented into pieces too small to be sedimented in the first slower spin. The second centrifugation produced 11.16 nmoles of *bo* and so it was presumed that the bulk of the *bo* did not remain in the supernatant after the first centrifugation. It was decided that the cells were insufficiently broken to release the membranes and so 0.1 g of lysozyme was added to the chamber during the next trial to aid the disruption of the cells. The resulting membrane suspension although darker (suggesting a higher oxidase concentration) contained a large amount of DNA and it was not possible to pipette the suspension. The oxidase concentration was not determined, as it was not possible to accurately dilute the sample. To overcome this 0.05 g of DNAase II was added to the chamber at the start of the next trial. This trial was unsuccessful. DNAase II cuts the 5' end of the DNA strand and is therefore not as efficient at shortening DNA as DNAase I which cuts at the ends and in the middle of the strands. For the second trial of DNAase, DNAase I was added to the chamber. DNAase requires the presence of a divalent cation in order to function (Sigma-aldrich, 2003) and so EDTA was omitted from the buffer for the next trial and 1 mM $MgSO_4$ was added to supplement the available divalent cations.

When the yield was adequate the method was applied to *Vitreoscilla* LB13. As the cells were harder to grow than *Escherichia coli* RG145 only 35 g were added to the chamber for the first trial. The final trial of RG145 cells produced a yield of 0.091 μ moles of membranous cytochrome *bo* 100 g^{-1} cells. A yield of 0.27 μ moles 100 g^{-1} cells was obtained from the first *Vitreoscilla* trial. The increase

in yield when the technique was applied to *Vitreoscilla* could be the result of reducing the quantity of cells added from 100 g to 35 g and therefore increasing the relative concentrations of lysozyme and DNAase. Familiarisation with the technique may also have increased the yield. There was a tendency to over estimate the concentration of *bo* present in the *Vitreoscilla* membranes because of changes in light scattering that caused the difference spectrum to slant. Also *Escherichia coli* RG145 does not produce cytochrome *bd* while *Vitreoscilla* does. The figure obtained for *bo* concentration in *Vitreoscilla* membranes would include cytochrome *bd*.

3.3.3 Urea/cholate wash

During the first run of the purification protocol (see Figure 3.1 Page 73) the urea cholate wash was omitted. It was thought that omitting this step might save time and avoid loss of product. When the product from this trial was applied to the DEAE column it failed to bind. Clearly the detergent extraction step had not successfully solubilised the membrane. This prevented the product from binding to the column. It was therefore necessary to include the urea cholate step in the protocol.

The purpose of the urea/cholate wash was to remove unwanted protein and leave the *bo* behind in the membranous fraction. During the first trial of the urea/cholate wash the quantity of cytochrome *bo* present fell by 67 %. The centrifugation steps were investigated to make sure that cytochrome *bo* was not being lost in the supernatant. The concentration of *bo* in the supernatants were measured, 2.43 nmoles were detected in the supernatant from the urea wash and 9 nmoles in the supernatant from the cholate wash. This alone does not account for the 0.128 μ mole loss over the whole manipulation. Overestimation of the *bo* present

in the membrane samples would account for some of the loss but it would appear that the enzyme was being degraded by a step in the protocol. Problems had been encountered with the centrifugation step. It was not possible to run the MSE 24 centrifuge at the correct speed and temperature because of problems cooling the rotor. For the second trial an ultracentrifuge was used (Beckmann L755) in the hope that a constant 4 °C would limit the loss. The second trial of the urea/cholate prewash produced similar results to the first (see Table 3. 2 Page 92) despite the loss in product the urea cholate wash still produced an increase in purity of the product.

Table 3.2 yield of cytochrome from the urea/cholate wash

Stage	Apparent Cytochrome <i>bo</i> μmol	Protein mg	Specific content (nmolmg^{-1})	% Yield cytochrome <i>bo</i>	Degree of purification
Initial quantities	0.243	1000	0.243	100	1
Supernatant from urea/cholate wash	0.0144	312.5	0.05	5.9	0.21
Pellet from urea/cholate wash	0.057	96	0.6	23.5	2.47

Only 5.9 % of the product was found in the supernatant but the pellet itself only contained 23 % of the original content. Therefore nearly 71 % of the product was lost during this procedure. The protein content of the product also dropped considerably during the wash and was not present in the supernatant. It was not possible to determine the cause of the loss of cytochrome. An experiment was conducted in which the enzyme was incubated in the presence of urea/cholate at concentrations used in the wash. The membranes were incubated for one hour in buffer, 5 M urea or 6.15 % cholate, the concentration of cytochrome *bo* was measured spectrophotometrically as previously described (see Materials and Methods Page 75). Little difference was seen between the membranes incubated in buffer and those incubated in urea or cholate.

3.3.4 Detergent extraction trials

Once respiratory membranes had been isolated the next step in the purification was to remove the enzyme from the membranes using a detergent (see Figure 3.1 Page 73). In order to maximise yield and minimise detergent used trials were run to determine the optimal concentration of detergent to use to extract the enzyme.

Table 3.3 Yield of cytochrome *bo* from detergent concentrations from 0.15 % to 2.00 % of final volume. Details of this experiment can be found in the Materials and Methods section Page 77.

Organism	Detergent %	Detergent (mg)	Pellet diameter (mm)	[<i>bo</i>]. (μM)
<i>Vitreoscilla</i>	0.15	1.5	3	0.29
<i>Vitreoscilla</i>	0.25	2.5	3	0.50
<i>Vitreoscilla</i>	0.50	5.0	3	0.63
<i>Vitreoscilla</i>	1.00	10	3	0.24
<i>Vitreoscilla</i>	1.50	15	2.5	0.49
<i>Vitreoscilla</i>	2.00	20	2.5	0.50
<i>E. coli</i>	0.15	1.5	11	Lost sample
<i>E. coli</i>	0.25	2.5	12	0.56
<i>E. coli</i>	0.50	5.0	12	0.75
<i>E. coli</i>	1.00	10	11	2.51
<i>E. coli</i>	1.50	15	7	2.15
<i>E. coli</i>	2.00	20	5	2.50

For the *Vitreoscilla* there was no significant increase in yield above 1.5 % detergent. The RG145 results showed no increase after 1 %. The detergent concentration was also varied according to protein concentration of the samples (Table 3.4 Page 90).

Table 3.4 Yield of cytochrome *bo* from detergent extraction trial, protein to detergent concentrations from a ratio of 1:1 up to 1:10. Details of this experiment can be found in the Materials and Methods section Page 77.

Organism	Detergent ratio	Detergent (mg)	Pellet diameter (mm)	[<i>bo</i>]. (μM)
<i>Vitreoscilla</i>	1:1	7.52	4	01.11
<i>Vitreoscilla</i>	1:4	1.88	6	00.36
<i>Vitreoscilla</i>	1:6	1.24	6	00.31
<i>Vitreoscilla</i>	1:8	0.94	6	00.28
<i>Vitreoscilla</i>	1:9	0.83	6	00.28
<i>Vitreoscilla</i>	1:10	0.75	6	00.26
<i>E. coli</i>	1:1	28.6	7.17	11.67
<i>E. coli</i>	1:4	7.14	14	04.04
<i>E. coli</i>	1:6	4.71	13	02.51
<i>E. coli</i>	1:8	3.57	14	01.29
<i>E. coli</i>	1:9	3.15	16	01.51
<i>E. coli</i>	1:10	2.85	16	01.26

The quantity of *bo* recovered from *Vitreoscilla* was similar for all ratios except 1:1 where the amount of *bo* extracted was considerably higher than the next highest detergent to protein ratio. The trial was rerun using just *Vitreoscilla* and including 1 higher detergent to protein ratio. Above a ratio of 1:1 there was no increase in the quantity of cytochrome extracted.

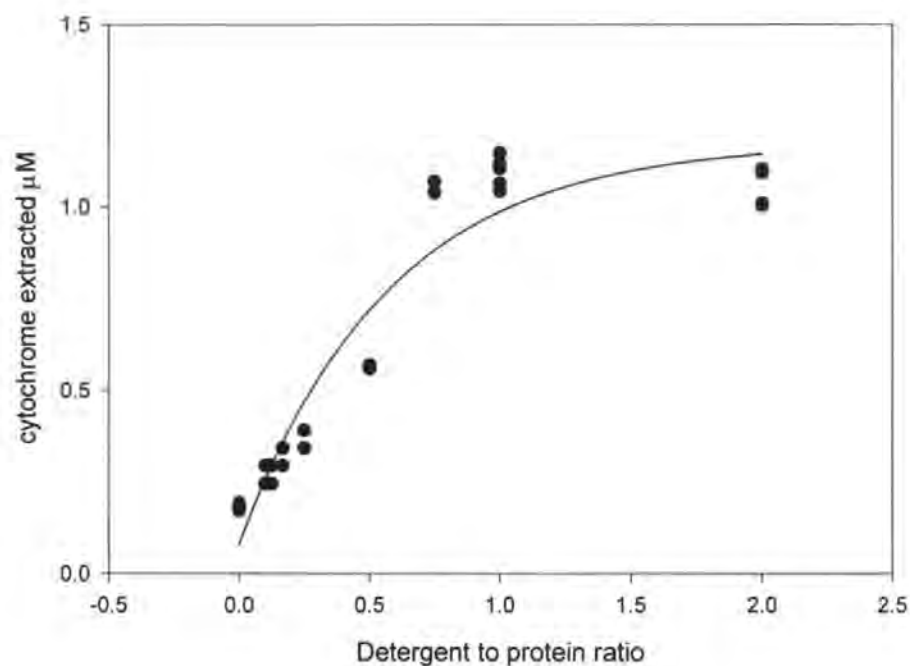


Figure 3.2 Cytochrome *bo* extracted from *Vitreoscilla* membranes at increasing detergent to protein ratios. A difference spectrum was measured and the extinction coefficient $20.5\text{mM}^{-1}\text{cm}^{-1}$ $\Delta_{560-580\text{nm}}$ was used to determine the quantity of *bo* extracted.

A detergent to protein ratio of 0.75 was chosen. This would both reduce the quantity of expensive detergent needed for each preparation and it was hoped would reduce the possibility of removing unwanted elements from the membranes such as flavoproteins. As octylglucoside was successful in extracting the enzyme from the membranes it was not thought necessary to trial any other detergents.

3.3.5 Quinol oxidation activity in the presence of KCN

Inhibition of the quinol oxidation activity of the membranes from LB13 by KCN was around 70 %. This indicates that 30 % of the activity measured is KCN insensitive and therefore likely to be caused by cytochrome *bd*. The extracted enzyme showed 100 % inhibition and so it can be assumed that the detergent either had not solubilised the *bd* and it was removed in the centrifugation step or that the octylglucoside inhibited the *bd*. If the *bd* were simply being inhibited by the detergent this might pose problems once the octylglucoside has been exchanged for Triton X-100 on the DEAE column. It was therefore necessary to test the purified enzyme for KCN sensitivity. The purified sample showed 100 % inhibition with KCN and so it is presumed that the purification process had successfully removed the *bd*.

3.3.6 DEAE Sepharose column

After extraction of the enzyme from the respiratory membrane further purification was achieved using a DEAE Sepharose column (see Figure 3.1 Page 73). The absorbance at 407 nm of each fraction was measured (see Figure 3.3 Page 94) and a difference spectrum of fractions 9, 22, 32, 41, 50 and 65 was taken. Fractions 24 to 61 were pooled. The quantity of *bo* in the sample was found to be 0.02 μ moles and the protein content was 11.5 mg.

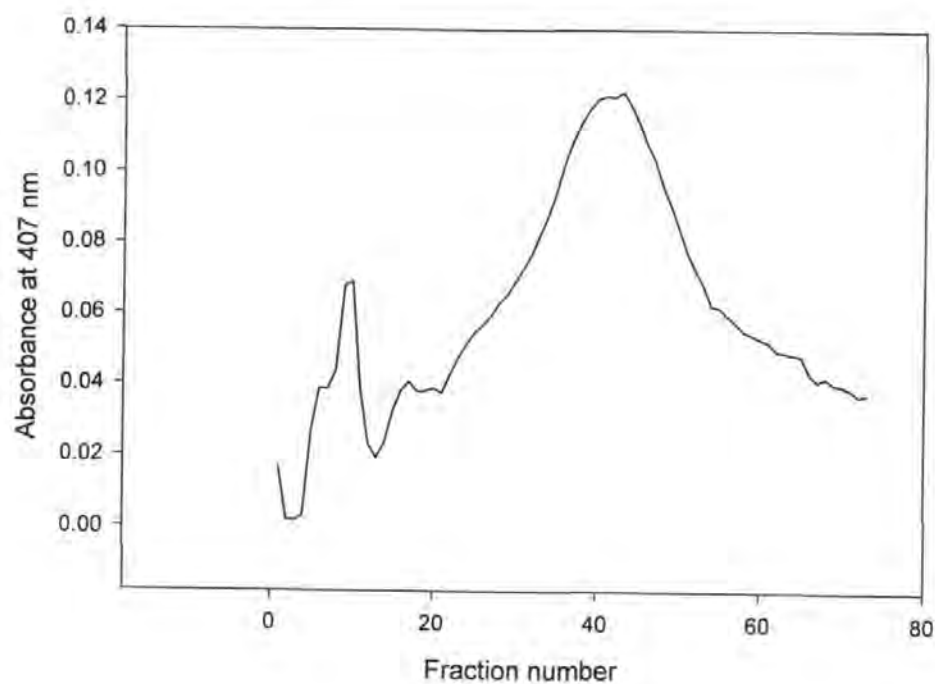


Figure 3.3 Elution profile from the DEAE Sepharose column. 8 min (2 ml) fractions were collected and the absorbance at 407 nm was measured (see Materials and Methods for details).

3.3.7 Hydrophobic interaction column

The final step in the purification was a hydrophobic interaction column (see Figure 3.1 Page 73). When the wash was run through the column the sample ran straight through. The sample was found to contain 0.005 μ moles of *bo* and 2.7 mg of protein after concentration to 1.5 ml. Although sample had been lost on the hydrophobic interaction column some purification had taken place. The loss of *bo* was considered too great compared to the purification gained and so in future preparations the hydrophobic column was omitted.

3.3.8 Overview of the purification process

Table 3.5 (Page 96) shows the quantities of cytochrome *bo* and protein at each stage of the purification process. The urea cholate wash step caused the largest loss in product, 77 % was lost at this stage whilst the purity was only improved 2.5 fold. The detergent extraction step provided the good increase in purity (2 fold) whilst only losing 10 % of the product. The DEAE column caused the loss of half the product but improved the purity 4 fold. Overall only 7 % of the cytochrome *bo* measured in the membranes was recovered by the end of the preparation. However it should be noted that the initial measurement of cytochrome *bo* includes cytochrome *bd*. Only 7 % of the cytochrome *bo* present at the beginning of the preparation was recovered. Georgiou *et al* purified cytochrome *bo* from *E. coli* RG145 and recovered 23.3 % of the product from membranes (Georgiou, Cokic *et al.*, 1988). *E. coli* RG145 over expresses cytochrome *bo* five fold and does not produce cytochrome *bd* (Au and Gennis, 1987), therefore the initial concentrations of heme *b* quoted do not contain a measurement of cytochrome *bd*.

Table 3.5 Changes in cytochrome *bo* quantities and purity over the whole purification process. The steps for the purification are described in Materials and Methods Page 75.

Stage	Apparent Cytochrome <i>bo</i> μ moles	Protein mg	Specific content nmoles mg^{-1}	% Yield cytochrome <i>bo</i>	Degree of purification
Membrane fraction	0.243	1000	0.243	100	1
After urea cholate wash	0.0572	96	0.6	23.54	2.5
After detergent extraction	0.0342	30.4	1.125	14.07	4.6
After DEAE column	0.0163	3.6	4.5	6.71	18.5

The hydrophobic interaction column had to be removed from the purification procedure because too much product was lost in this step, purity had to be sacrificed in order to obtain sufficient product. Had more product been recovered from the previous stages of the purification this may not have been a problem. The biggest loss was in the urea cholate step where 70 % of the cytochrome *bol/bd* was lost. If this problem could have been overcome then the overall yield would have been greatly improved. Unfortunately it was not possible to determine where in this step the product was being lost.

Overall the purification was successful; sufficient product was recovered to perform measurements of activity although had the study gone on to include insertion of the enzyme into vesicles then the process may have needed to be refined to obtain more product.

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Chapter 4

The effect of UV-A on the growth of *Vitreoscilla*

4.1 Introduction

Of the radiation emitted by the sun 3% is of a wavelength less than 390 nm (the UV region of the spectrum). Because of absorption by the Earth's atmosphere only a fraction of this harmful radiation reaches the surface of the planet. Ultraviolet light is classified into three bands according to its effects. Short wavelengths between 100 and 280 nm are termed UV-C. These short wavelengths are not naturally seen at the surface of the earth because they are quickly absorbed within a few hundred meters of the atmosphere (Gascon, Oubina et al., 1995). Therefore, bacteria and other organisms do not normally encounter this form of ultraviolet light. Having not evolved a defence mechanism, bacteria are very susceptible to damage by UV-C and it is therefore commonly used as an antimicrobial agent to sterilise air and water (Liltved and Landfald, 2000).

Ultraviolet light of wavelengths between 280 and 315 nm is termed UV-B. Although most UV-B is absorbed by the atmosphere, some still reaches the Earth's surface and is responsible for much of the cellular damage associated with sunlight. Absorption of UV-B causes direct damage to DNA. The most common reaction is the formation of cyclobutylthymine dimers between adjacent thymine residues on the DNA strand (Setlow and Setlow, 1962). The repair mechanism used by many bacteria to combat the effects of UV-B is prone to error and is the cause of many of the UV induced mutations (Prescott, Harley et al., 1990).

The majority of solar UV radiation reaching the Earth's surface is of wavelengths between 315 and 400 nm and is termed UV-A. Many cellular components absorb UV-A, and reactive oxygen intermediates such as singlet oxygen, superoxide, and peroxy/hydroxyl radicals may be generated as a result. These reactive oxygen species can cause damage to DNA, proteins and membrane lipids and can

ultimately cause the death of the cell (Jagger, 1983). Aerobic bacteria have evolved enzymes to combat this oxidative damage, for example superoxide dismutase and catalase, as well as enzymes which repair damage caused to DNA (Halliwell and Gutteridge, 1999).

Research into the susceptibility of bacteria to UV damage has concentrated mainly on species that would naturally be exposed to higher levels of UV, for example organisms which inhabit surfaces or surface water. These organisms would be expected to have some resistance to UV. Examination of the effect of different UV wavelengths on the survival rates of *Staphylococcus aureus* (mainly associated with the skin of warm blooded vertebrates) showed that the bacterium is fully resistant to UV-A. Over a 4 hour period of exposure to UV-A the *S. aureus* showed no drop in viability. However when exposed to a broad band UV-B lamp filtered by Perspex to remove UV-C for 3 hours a 99% reduction in viability was observed. Although the exposure was not quantitated. (Eladhami, Daly et al., 1994).

Although these bacteria have evolved to overcome the problems associated with UV light the addition of an extra stress can have an effect on their ability to survive UV damage. Martin *et al.* compared the ability of two saltern bacteria, a moderate halophile (*Halomonas elongata*) and an extreme halophile (*Halobacterium salinarum*) to withstand UV exposure at increasing sodium chloride concentrations (Martin, Reinhardt et al., 2000). The moderate halophile was resistant to UV-A and B at 0.05 M sodium chloride but at 4.3 M sodium chloride showed a 10 000 fold decrease in colony forming units after 120 min of exposure to UV-A and UV-B (19.6 kJm⁻²). In contrast the extreme halophile showed no drop in viability at 0.05 or 4.3 M sodium chloride.

Few studies have looked at the ability of bacteria that are not usually exposed to UV-A to withstand exposure. Fernandez & Pizarro looked at *Pseudomonas aeruginosa* (a soil associated organism) and found a survival rate of only 20 % when the organism was exposed to 120 kJm⁻² of UV-A over an hour (Fernandez and Pizarro, 1996). Fernandez & Pizarro compared the sensitivity of the *Pseudomonas* with a strain of *Escherichia coli* (a gut associated organism). They found that the *E. coli* was able to withstand a level of UV-A which caused severe damage to the *Pseudomonas* but that a lag in growth was observed post irradiation. The elimination of oxygen during exposure to UV-A prevented the bacteria from being damaged, indicating that oxidative stress was indeed responsible for UV-A lethality.

During routine culture, broths inoculated with *Vitreoscilla* that were exposed to diffuse light failed to grow; significant growth was only observed if cultures were set up in the evening. *E. coli* cultures grown under the same conditions on the window bench grew normally, reaching maximum density in the expected time and displaying no inhibition. Further investigation suggested that the organism is light sensitive. Possible causes of the light sensitivity were investigated and the role of reactive oxygen species was explored. The involvement of the electron transport chain and/or cytoplasmic haemoglobin (which *Vitreoscilla stercoraria* is known to contain high levels of) (Dikshit and Webster, 1988) in the generation of reactive oxygen species was explored. Most of this work has been published and a reprint can be found in the appendix.

4.2 Materials and methods

All microbiological media were obtained from Oxoid Ltd (Basingstoke, UK). All reagents were obtained from Sigma (Poole, UK) unless otherwise stated.

4.2.1 Bacterial strains and culture conditions

Escherichia coli K12 (ATCC 23716) was used as a control organism. *Vitreoscilla stercoraria* strain LB13 was isolated directly from the environment (as described in Chapter 2, Page 38). *Vitreoscilla stercoraria* strain LB13A was isolated during a light exposure experiment (see Materials and Methods Page 104) and originated from a culture of LB13. *Vitreoscilla stercoraria* strain C1 (ATCC 13981) was kindly donated by Dr Dale Webster (Illinois Institute of Technology).

General culture maintenance conditions for *Vitreoscilla* are described in Chapter 2, Page 39. Bacterial cells were incubated 25°C with vigorous shaking for C1 and *E. coli* K12, and no shaking for LB13 and LB13A, in modified tryptone yeast (MTY) broth. MTY broth contained 10 g l⁻¹ tryptone (Oxoid L42); 10 g l⁻¹ yeast extract (Oxoid L21); and 2.5 g l⁻¹ NaCl. The pH was adjusted to pH 7.8 with NaOH. All cultures were grown in the dark except LB13A, which was grown under a low level of full spectrum natural light in a Fisons Fitotron 600 growth cabinet (Fisons, Loughborough, UK).

4.2.2 Cell irradiation procedure

All manipulations were performed under low light conditions. Cultures of *Vitreoscilla* were grown for two days before exposure and *E. coli* K12 was grown overnight. The cultures were centrifuged at 2000 *g*_{av} in an MSE Super Minor, bench top centrifuge for 30 min. The supernatant was removed and the weight of

the pellet determined. The cells were resuspended to 0.1 gml^{-1} in cool physiological saline solution (NaCl , 9 gl^{-1}); dilutions were made by trial and error in order to achieve an initial count of $5 \times 10^7 \text{ cfuml}^{-1}$. Ten millilitres of each appropriately diluted bacterial suspension was placed in a tissue culture vessel (80 cm^2 Nunclon flasks, Nalge Nunc International, Denmark or 25 cm^2 Falcon flasks, Becton Dickinson Europe, Meylan Ced  x, France). The bacterial suspensions were exposed to UV-A with a long wavelength (365 nm) UV lamp (model UVLS-28, UVP Inc., Upland, California), positioned 0.2 m above the samples. The intensity of UV-A and dosage received by the samples during the experiment was measured using an SR9910 spectroradiometer (Macam Photometrics Ltd, Livingston, UK). The flasks were kept on ice whilst under the lamp and were shaken at 100 rpm. Samples were removed after every hour and a viable count was performed on these. The position of the culture vessels under the UV lamp was changed after every hour.

4.2.3 Isolation of respiratory membranes and a cytosolic extract containing bacterial haemoglobin from LB13 and LB13A

Large scale growth of *Vitreoscilla* and isolation of respiratory membranes is described in Chapter 3 Page 74 and 75 respectively Bacterial haemoglobin was isolated by retaining the supernatant from the respiratory membrane isolation.

4.3 Results

4.3.1 Initial light sensitivity experiment

Vitreoscilla strain LB13 was grown on MTY agar for 48 hours and then inoculated into MTY broth. The culture was grown for a further 48 hours and then 100 µl was inoculated into each of 123 universal bottles containing 10 ml of MTY broth. The universal bottles were incubated at 25 °C in a plant growth cabinet (Sanyo MLR-350 HT; Sanyo Gallenkamp plc, Loughborough, UK) under one of four light/dark regimes: (1) light, running with 12 × 40 W fluorescent lamps, for 96 hours; (2) dark for 96 hours; (3) light for the first 24 hours, then dark; and (4) dark for the first 24 hours, then light. At intervals bottles were removed and the absorbance of the culture at 590 nm was measured. A viable count was performed on each bottle every 24 hours from 24 hours up to 96 hours. A sample (100 µl) of the culture was removed and diluted with fresh MTY broth. The diluted bacteria (100 µl) were spread onto MTY agar plates and were incubated at 25°C for 48 hours under the light conditions under which they had been growing. The number of colonies was then counted.

Cultures grown in the light for the length of the experiment showed no growth measurable by optical density Figure 4.1 Page 107. Cultures grown without light for the length of the experiment grew following a normal growth curve, reaching a maximum measurable density after 40-60 hours. *Vitreoscilla* cultures reaching a certain density naturally begin to flocculate, making optical density measurements unreliable. Cultures that were kept dark for 24 hours then moved to the light showed an initial increase in optical density (whilst dark), a lag and then a fall after

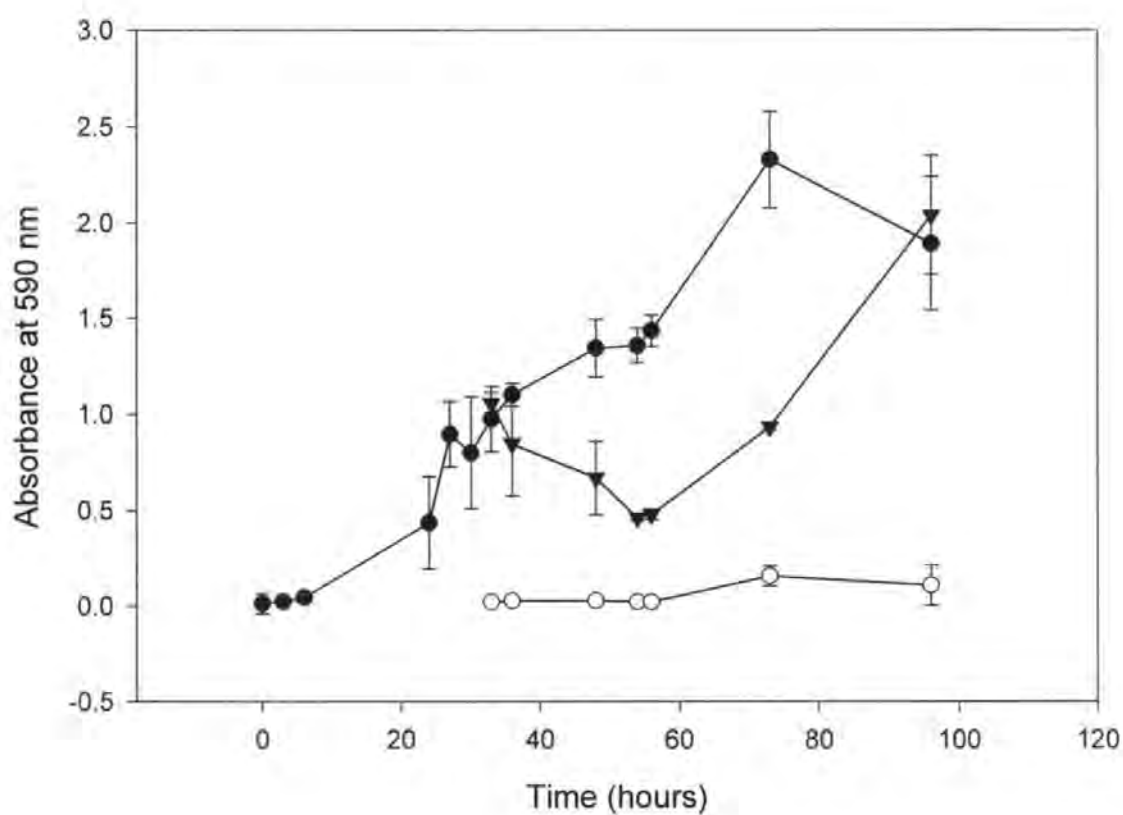


Figure 4.1 The effect of light on the growth of *Vitreoscilla* strain LB13. Each point represents the average absorbance of cultures from 3 bottles. O Light for 96 hours ● Dark for 96 hours ▼ dark for 24 hours then light. Cells were grown in universal bottles in 10 ml of broth at 25°C in a Sanyo plant growth cabinet. At times indicated 3 bottles from each light regime were removed and the absorbance measured.

they were moved to the light. These cultures did, however, recover; after 20 hours in the light the optical density once again began to rise and after 100 hours had reached a similar density to the cultures that had been kept in the dark. The cultures that were kept light for 24 hours and then changed to dark showed a very slight rise in optical density after 70 hours but did not reach a density comparable with the cultures that had been kept dark throughout.

Only the plates from the bottles that had been kept dark and the bottles moved from dark to light produced countable numbers of colonies. The counts were variable with numbers ranging from 35×10^6 to 260×10^6 CFU ml⁻¹ for plates at 48 hours for the dark cultures and 34×10^6 to 270×10^6 CFU ml⁻¹ at 48 hours for the cultures that were moved from dark into light. Variation in the viable counts of the cultures was consistent with the variation in optical densities for those cultures. At 48 hours, on the plates from the cultures incubated in the light throughout two colonies were detected that appeared to be light resistant. On examination the bacteria resembled (size, shape and colony morphology) *Vitreoscilla* LB13 and were not thought to be contamination. One of these colonies was removed and restreaked onto fresh agar. The new strain was named LB13A and stored for further work.

4.3.2 Exposure of two strains of *Vitreoscilla* and one strain of *E. coli* to UV-A

Although the above experiment demonstrated the sensitivity of *Vitreoscilla* it was desirable to narrow the wavelength range to which the organism was exposed in order to determine which part of the spectrum was responsible for the damage. A lamp was available that emitted UV-A and so this was used for the next experiment. A wild type strain of *E. coli* (K12) was tested along side the three available strains of *Vitreoscilla* (LB13, LB13A and C1) for comparison. The

organisms were exposed to UV-A as described in the Materials & Methods section (Page 104). Samples were removed from the flasks every hour and viable counts were performed. Figure 4.2 Page 110 shows the resulting viable count versus dose for this experiment. A lag in response was seen for the first hour of exposure but after 2 hours the two wild type strains of *Vitreoscilla* had a fivefold reduction in viability whilst the *E. coli* strain and the newly isolated LB13A showed no drop in viability. The LD₅₀ for LB13 and C1 were similar at about 15 kJ m⁻² whilst both *E. coli* K12 and LB13A were not sensitive to UV-A. The viability of the two wild type strains continued to drop although not as sharply as during the second hour of exposure. *E. coli* and LB13A once again showed no drop in viability.

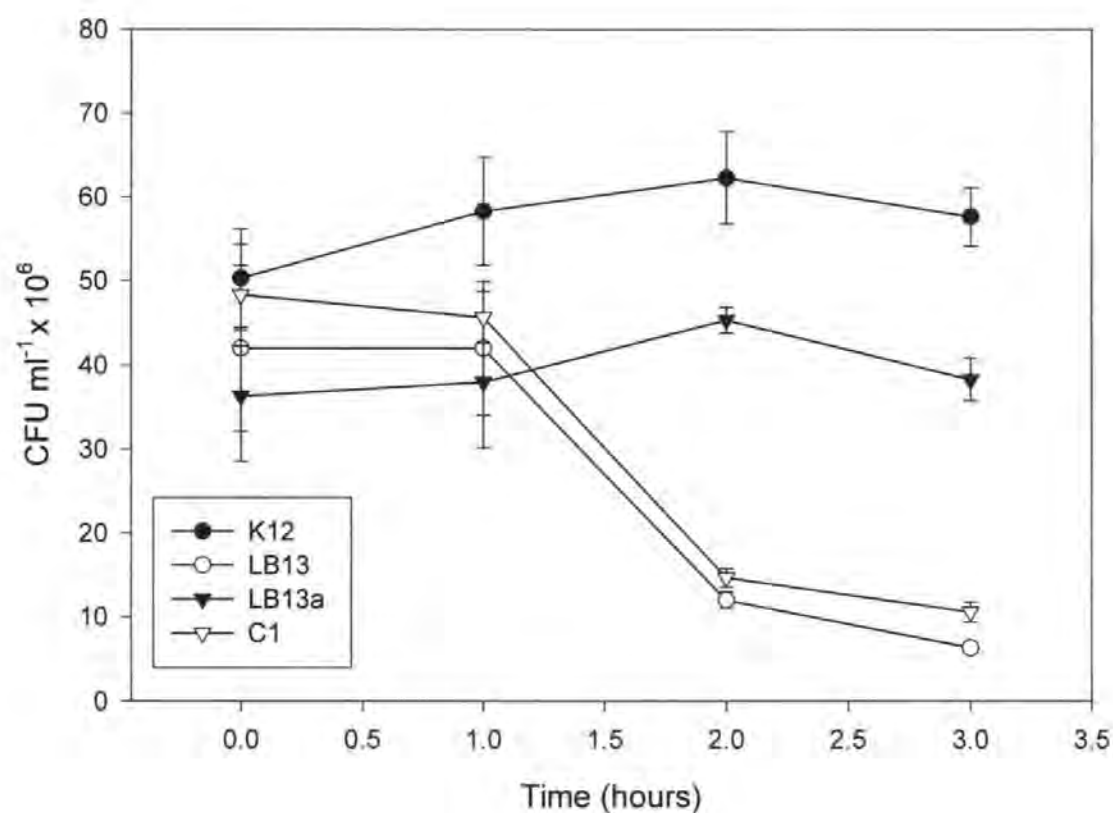


Figure 4.2 The effect of UV-A irradiation at $91 \text{ Jcm}^{-2} \text{ hour}^{-1}$ on the number of colony forming units per ml of a strain of *E.coli* and three strains of *Vitreoscilla* (as indicated). Cells were exposed for three hours whilst gently shaking on ice; samples were taken every hour diluted and plated onto agar at which time the samples positions under the light were changed. Each point represents the average count from three flasks plus or minus one standard deviation.

4.3.3 UV-A exposure in the absence of O₂

It was postulated that reactive oxygen species were responsible for damage caused by UV-A and ultimately the light sensitivity of *Vitreoscilla*. In order to verify this LB13 samples were exposed to UV-A in the presence and absence of O₂. The experiment was conducted essentially as described above (see also Materials & Methods, Page 104). To eliminate O₂ the culture flasks were flushed with nitrogen for 1 min before exposure (oxygen free, BOC, Guildford, Surrey). When samples were removed for plate counts the flasks were once again flushed with nitrogen. Figure 4.3 Page 112 shows % survival versus UV-A dose. In both the presence and absence of O₂ a drop in viability after a dose of 91 Jcm⁻² was seen. At dosages greater than 91 Jcm⁻² the viability of samples that had been deprived of oxygen stabilised. However, in the presence of oxygen viability continued to decline at dosages greater than 91 Jcm⁻². This experiment was repeated a number of times but was difficult to reproduce, probably because of variation in the available oxygen between experiments and also the experimental conditions were unfavourable to the organism (see Discussion, Page 120).

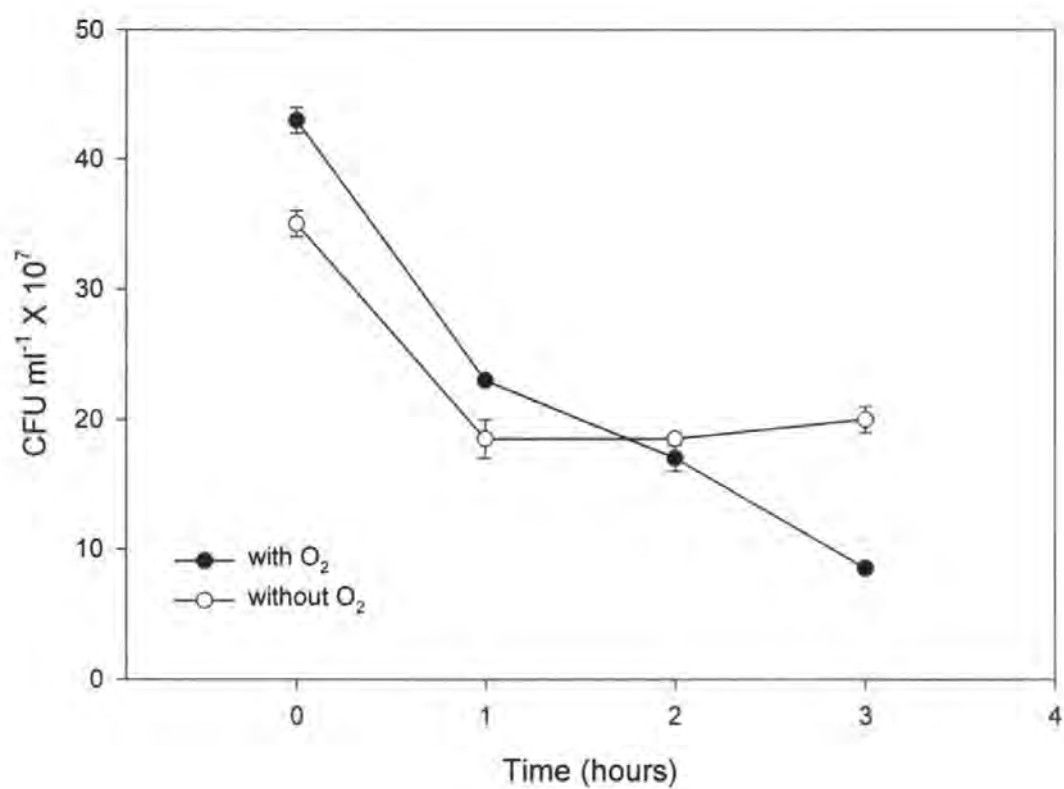


Figure 4.3 The effect of UV-A irradiation at $91\text{Jcm}^{-2}\text{hour}^{-1}$ on LB13 in the presence and absence of oxygen. The oxygen free samples were gassed with nitrogen for one min. before exposure. Every hour a sample was taken out and the cells were gassed again. The samples were diluted and plated and a viable count was performed.

4.3.4 Protection from UV-A damage by superoxide dismutase and/or catalase

If reactive oxygen species are responsible for the UV-A sensitivity of *Vitreoscilla* then it might be possible to protect the bacteria by the addition of superoxide dismutase and/or catalase. Therefore *Vitreoscilla* were exposed to UV-A in the presence of superoxide dismutase (SOD) and/ or catalase to determine whether or not any protection was apparent. Exposure was conducted as described in Materials and Methods section Page 104. Superoxide dismutase (750 units Sigma) and/or catalase (1 μg in 100 μl Sigma C₄₀ 2000-5000 units ml^{-1} protein) was added to the flasks prior to illumination. Figure 4.4 Page 114 shows the % survival compared to the light flasks for each treatment. The addition of superoxide dismutase or catalase provided a marked protection. The addition of both cat. and SOD provided almost full protection from the effects of UV-A.

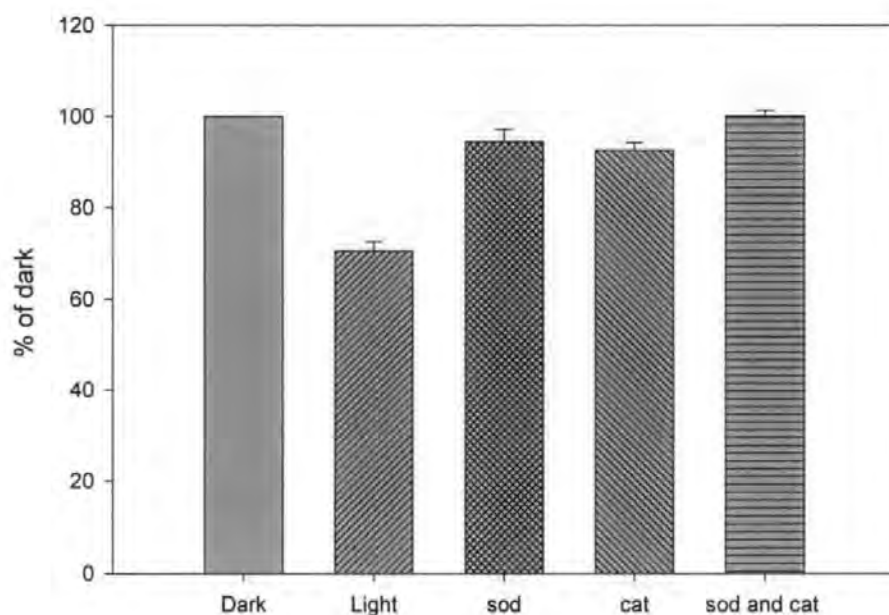


Figure 4.4 The effect of the addition of superoxide dismutase and/or catalase on the light sensitivity of *Vitreoscilla* LB13. Exposure was conducted as described in the methods (Page 104) Superoxide dismutase (750 units Sigma S 4636) and/or catalase (1 μ g in 100 μ l sigma C40 2000-5000 units ml^{-1} protein) was added to the flasks prior to illumination. Each bar represents the average of three experiments with three flasks for each condition.

4.3.5 Comparison of respiratory membranes and cytosolic extracts from LB13 and LB13A

It was possible that the chromophore responsible for the light sensitivity of *Vitreoscilla* could be identified by comparison of the chromophore content of LB13 with that of LB13A, the photo sensitive strain derived from it. The assumption was that exposure of LB13 to light had selected for a mutant that lacked the ability to produce this chromophore. Respiratory membranes from two strains were isolated and compared for differences and the two strains were tested for both haemoglobin production and the presence or absence of complex I.

4.3.5.1 Examination of respiratory membranes by difference spectroscopy

Membranes were isolated as described in Chapter 3 Material and Methods section Page 75. Absorbance was measured from 400 nm to 700 nm for both the reduced and oxidised membranes from each strain, and the oxidised spectrum was subtracted from the reduced to produce a difference spectrum Figure 4.5 Page 116. Membranes from both strains showed evidence of the presence of both terminal oxidases *bo* and *bd* as indicated by the haems *b* alpha absorption band at 560 nm and by small absorption features between 600 and 700 nm (Georgiou and Webster, 1987). Initial experiments looked promising showing a difference in flavin content between the two strains. The presence of flavins in LB13 is indicated by the minimum in the spectrum at 460 nm. This result, however could not be reproduced.

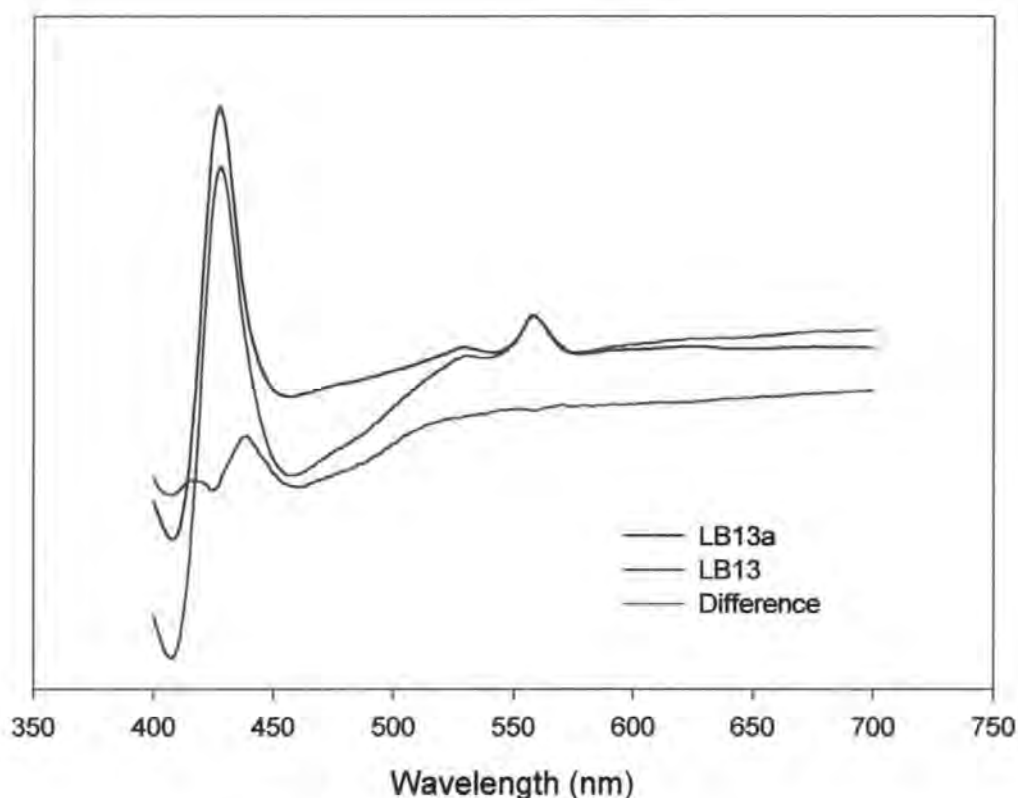


Figure 4.5 Difference spectra of membranes from LB13 and LB13A, partially solubilised with octylglucoside at a ratio of 0.75 detergent to protein. Membranes were partially solubilised with octylglucoside (n-octyl β -D-glucopyranoside; Alexis Biochemicals, San Diego CA) at a ratio of 0.75 detergent to protein (by weight). The supernatant, i.e. the cytosolic extract, containing the bacterial haemoglobin was retained. Membranes (100 μ l) were added to potassium phosphate buffer pH 7.5 (400 μ l) difference spectra were recorded. Potassium dithionite was used as the electron donor. Spectra were normalised using the difference between the absorbance at 560 nm minus the absorbance at 580 nm.

4.3.5.2 Comparison of cytosolic extracts

Vitreoscilla are known to produce bacterial haemoglobin (Wakabayashi, Matsubara et al., 1986). It is possible that a difference in the production of this haemoglobin could be responsible for the difference in sensitivity to UV-A of the two strains. The quantity of haemoglobin in the cytosolic extracts from each strain was therefore measured. The cytosolic extract was isolated as described in the Material and Methods section Page 105. Neat extract was reduced using dithionite and CO was bubbled through it for 1 min. The spectra of both the reduced and CO bound extracts were taken and the difference calculated (Figure 4.6 Page 118 shows the resulting spectra). The quantity of haemoglobin in the extract from each strain was calculated using the extinction coefficient $\epsilon_{419-436 \text{ nm}} = 274 \text{ mM}^{-1} \text{ cm}^{-1}$ (Dikshit and Webster, 1988). The first preparations of cytosolic extract produced samples with different concentrations of haemoglobin. LB13 contained 93 nmoles of haemoglobin per gram of protein and LB13A contained 50 nmoles of haemoglobin per gram of protein. Aeration rates for these preparations could have been improved; there was possibly an uneven delivery of air to the flasks. This is problematic when measuring the relative concentrations of haemoglobin in the cytosol of the strains as it has been reported that the production of haemoglobin is stimulated by low oxygen conditions (Joshi and Dikshit, 1994) Therefore the preparation was repeated. The new extract was centrifuged at 100 000 g for 1 hour in a Beckman TL-100 benchtop ultracentrifuge in order to remove any membrane vesicles and so improve the quality of the spectra. The content of haemoglobin was found to be 57.22 nmoles per gram of protein for LB13 and 40.33 nmoles per gram of protein for LB13A . It is clear, therefore, despite the variations, that both strains produce haemoglobin.

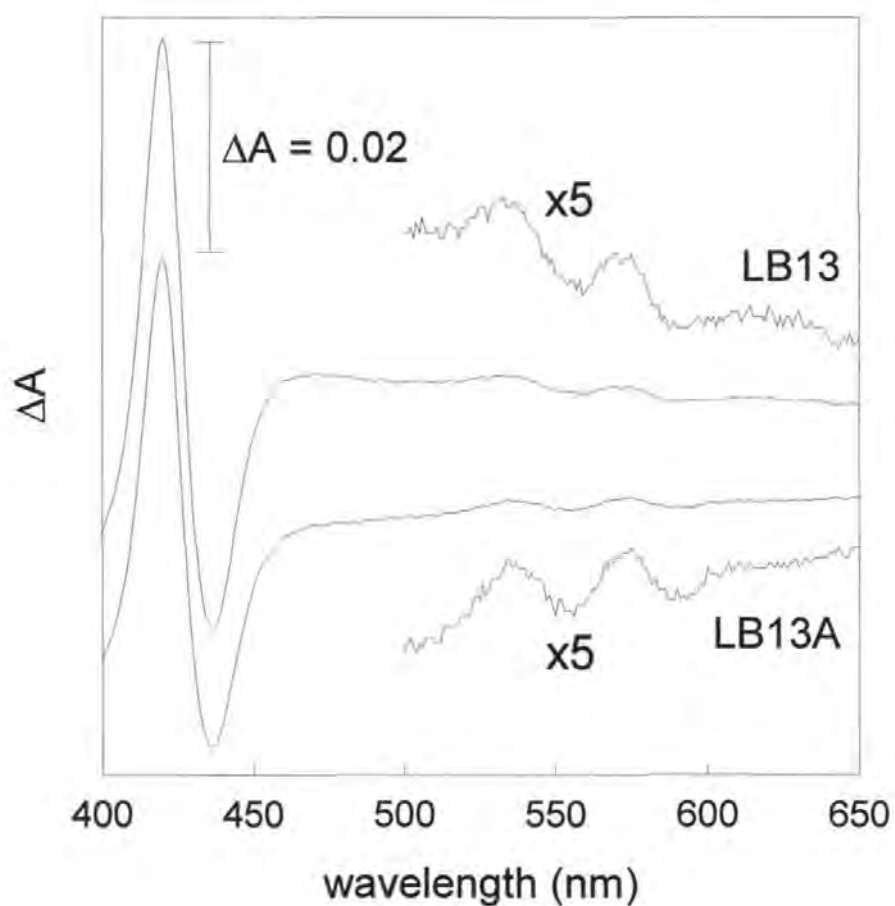


Figure 4. 6 The Co binding spectra of cytosolic extracts from LB13 and LB13A. The cytosolic extract was isolated as described in the Material and Methods section Page 101. Neat extract was reduced using dithionite and CO was bubbled through it for 1 min

4.3.5.3 Presence or absence of complex 1

As the concentration of haemoglobin was found to be similar and at this time the difference spectra had shown a difference in flavin content between the two strains, flavoprotein was examined as a possible source of UV-A sensitivity. The membranes of the two strains were examined for the presence of Complex 1. NADH oxidation by membranes from both LB13 and LB13A was tested for sensitivity to rotenone in order to determine the presence or absence of Complex I. Membranes (2 μ l or 5 μ l) from LB13 or LB13A, respectively, were added to 500 μ l of potassium phosphate buffer pH 7.5. To the cuvette was added 5 μ l of 1 mgml⁻¹ rotenone in ethanol, 5 μ l of ethanol or 5 μ l of water. The reaction was started by the addition of 5 μ l of 16 mM NADH and the rate of decrease in absorbance at 340 nm was monitored. A straight line was fitted to the first two min of each time course and the slope of the line used to determine the rate of oxidation.

The membranes from LB13 showed 65 % \pm 10.77 (\pm SEM, $n = 3$) inhibition from rotenone whilst the LB13A membranes showed only 40 % \pm 14.29 (\pm SEM, $n = 3$). However there was a lot of variation between replicates and a single factor analysis of variance showed no significant difference between the two sets of results, either way it is clear that both strains possess Complex I.

4.4 Discussion

4.4.1 Photosensitive growth of *Vitreoscilla* LB13

This work began with the observation that cultures of *Vitreoscilla* strain LB13 failed to grow when they were incubated close to an external window. This photosensitive growth was demonstrated in a first experiment in which the organism was exposed to UV together with visible light in a plant growth cabinet. It was not possible to define the exact dose of each component that the cultures received in this experiment.

As expected, growth in the cultures kept in the dark was normal (Figure 4.1 Page 107), but there was no growth in those kept in the light (not shown on figure). The principal interest is in the behaviour of the cultures kept under the dark/light regime. Growth in the 'dark/light' cultures was normal in the dark, but in the light, after a lag of about 8 h, cell density began to decrease. Hence, the light did not simply have a bacteriostatic effect. However, after about 40 h in the light growth recovered showing that the photosensitivity of *Vitreoscilla* LB13 can be selected out by growth of the organism under light. This was further confirmed as follows. At 48 h when 0.1 ml of a 10^{-5} dilution (in fresh MTY broth) of culture that had been kept in the dark was spread onto MTY agar and incubated for 24 h in the dark at 25 °C, 121 ± 70 (\pm SEM, $n = 3$) colonies were detected. This is equivalent to $121 \pm 70 \times 10^6$ colony-forming units per millilitre (cfuml⁻¹). In contrast, for the culture that had been exposed to light a total of only two colonies were detected when 0.1 ml of neat culture was spread in triplicate onto MTY agar and incubated under the same conditions in the light. This is equivalent to only 7 ± 7 cfuml⁻¹ (\pm SEM, $n = 3$). One of these two photo-insensitive strains was retained and designated LB13A.

Figure 4.2 (Page 110) shows the percentage survival of each of the four strains against UV-A dose. *Vitreoscilla* LB13 is clearly UV-A sensitive with an LD₅₀ of about 15 kJ m⁻². *Vitreoscilla* C1 behaved in a similar way, but both *E. coli* K12 and *Vitreoscilla* LB13A were UV-A insensitive, as expected. Both *Vitreoscilla* LB13 and C1 showed a threshold at about 9 kJ m⁻² below which they were insensitive to UV-A, at least under the conditions of this experiment.

4.4.2 Protection of *Vitreoscilla* LB13 against photodamage by UV-A

While DNA absorbs, and hence can be directly damaged by exposure to UV-C, and to a lesser extent UV-B (Jagger, 1983) this is not the case for UV-A. Hence, the observation that *Vitreoscilla* LB13 is sensitive to UV-A suggests an indirect mechanism for the photodamage. One possibility is that this could involve reactive oxygen species such as the superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), generated through the reaction of an excited chromophore with molecular oxygen. This was investigated by supplementation with the enzymes superoxide dismutase (SOD) and catalase (cat.), during the exposure of *Vitreoscilla* LB13 to UV-A. Figure 4.3 (Page 112) shows that both SOD and Cat., separately or in combination, provided significant protection to the organism against damage by UV-A.

4.4.3 Comparison of the bacterial haemoglobin content of *Vitreoscilla* LB13 and LB13A

Once the sensitivity of *Vitreoscilla* LB13 to UV-A was established the possibility arose that the chromophore responsible could be identified by comparison of the chromophore content of LB13 with that of LB13A, the photo-insensitive strain derived from it. A characteristic of *Vitreoscilla* is the presence of high levels of bacterial haemoglobin in the cytosol (Dikshit and Webster, 1988). CO-binding

spectra for cytosolic extracts from both LB13 and LB13A indicated the presence of haemoglobin (Figure 4.4, Page 114). In both cases bacterial haemoglobin is clearly present, hence eliminating the possibility that the absence of this chromophore accounts for the photo-insensitivity of LB13A. The levels of haemoglobin observed here (0.6-0.8 nmol/g wet weight) are somewhat lower than those reported for *Vitreoscilla* C1 (e.g. 30 nmol/g wet weight); (Dikshit and Webster, 1988). However, complete extraction of the haemoglobin is unlikely to be achieved with the method used here, and in any case the yield of haemoglobin is known to be highly dependent on growth conditions, a 20-40 fold higher yield being obtained after growth under hypoxic conditions (Boerman and Webster, 1982).

4.4.4 Comparison of the redox-active chromophore content of membranes from *Vitreoscilla* LB13 and LB13A

No major difference in the redox-active haem-chromophore content of the membranes was detected by difference spectroscopy (Figure 4.5 Page 116). When the difference spectra are normalised and one subtracted from the other, the difference below about 520 nm is dominated by a feature consistent with the bleaching on reduction of a flavin (Figure 4.5 Page 116). Note that the smaller feature (with a maximum at about 438 nm) superimposed on this is probably an artefact of the normalisation caused by a slight difference in the ratio of cytochrome *bo* to cytochrome *bd* in the two membrane preparations; their ratio is likely to be sensitive to slight differences in growth conditions (Kita, Konishi et al., 1986).

4.4.5 Flavins as chromophores

The potential role of flavins as chromophores involved in the lethal and sub-lethal effects of near-UV on bacteria has been discussed before (Jagger, 1983).

Oxidized riboflavin, for example, has an absorption maximum (375 nm) that closely matches the UV-A used here (365 nm). The absorption of UV-A by a flavin could lead to its photoreduction (Salet, Land et al., 1981; Traber, Kramer et al., 1982), which could in turn could cause direct damage by abstraction of electrons from components in its immediate environment. Alternatively this could cause indirect damage via the generation of superoxide (by donation of an electron to O₂) (Vaish and Tollin, 1971), which appears to be the case here. Flavins are common components of electron transfer complexes. One possibility here is the proton-pumping NADH:Q oxidoreductase (NQR-1), which contains FMN. However, membranes from LB13A showed NADH oxidase activity that was partly sensitive to rotenone indicating that NQR-1 was still present in this photo-insensitive strain.

4.4.6 Comparison of *Vitreoscilla* sensitivity with that of other bacteria

There has been a brief report of the photoinhibition of respiration in *Vitreoscilla stercoraria* (Ninnemann, 1972). In this work the rates of oxygen consumption by starved cells were measured after exposure of these to blue light (400 nm). A dose-dependent decrease in rate was observed, with 150 of about 1000 kJ m⁻² (i.e. 10 minutes at 1.8x10⁶ erg cm⁻² s⁻¹). It was implied in this work that the terminal oxidase cytochrome *bo* was the target for the photoinhibition, but no evidence for this was presented. The work presented here shows that cytochrome *bo* is unlikely to be the chromophore involved, but it could be a target for indirect damage. Whether or not the photoinhibition of respiration can in itself account for the killing effect of UV-A seen here (Figure 4.2 Page 110) remains to be seen.

Direct comparisons between the sensitivity of *Vitreoscilla* and other bacteria are difficult to make because of the different irradiation regimes that have been used. Joux *et al* looked at responses of marine bacteria to UV-B and found that with an LD₅₀ of about 100 kJm⁻² *Vibrio natriegens* was the most sensitive (Joux, Jeffrey *et al.*, 1999). Degiorgi *et al* looked at three bacteria and found that the most sensitive to UV-B was *Pseudomonas aeruginosa* with an LD₅₀ of about 20 kJm⁻² (Degiorgi, Fernandez *et al.*, 1996). The amount of UV-A that bacteria can survive is likely to be higher than UV-B because UV-A does not cause direct DNA damage. Tyrell reported LD₅₀ values (for *E. coli*) of 50 kJm⁻², 250 kJm⁻² and 1200 kJm⁻² for wavelengths of 313 nm, 334 nm and 365 nm respectively (Tyrell, 1985). The LD₅₀ for *Vitreoscilla stercoraria* UV-A exposure was found to be 15 kJm⁻² making it particularly sensitive compared to other bacteria.

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Chapter 5

The effect of sodium ions on the activity of cytochrome *bo* and *bd* in respiratory membranes and purified cytochrome *bo* from *Vitreoscilla* isolates LB13 and C1

5.1 Introduction

There have been reports that an alkotolerant/halotolerant strain of *Bacillus* (Semeykina, Skulachev et al., 1989) and *E. coli* grown at alkaline pH (Avetisyan, Dibrov et al., 1989) utilise a sodium pumping terminal oxidase. Neither of the enzymes responsible were identified. *Vitreoscilla* is therefore the only bacterium that has been reported to possess a sodium pumping terminal oxidase that has been identified and partially characterised (Bassey, Efiok et al., 1990a; Bassey, Efiok et al., 1992; Bassey, Efiok et al., 1990b; Park, Moon et al., 1996). In 1990 Bassey *et al* first reported the generation of a sodium gradient by the respiratory chain of *Vitreoscilla* (Bassey, Efiok, and Webster, 1990b). Bassey *et al* demonstrated that the formation of $\Delta\psi$ was directly coupled to respiratory-driven Na^+ extrusion using the following experimental evidence: there was a correlation between the kinetics of respiratory-driven Na^+ extrusion and $\Delta\psi$ formation; monensin (a sodium/hydrogen ionophore) caused the collapse of $\Delta\psi$; and the protonophore 3,5-di-tert-butyl-4-hydroxybenzaldehyde (DTHB) caused a transient collapse of the gradient followed by the stimulation of Na^+ extrusion and recovery of the $\Delta\psi$. There was no correlation between $\Delta\psi$ and H^+ fluxes (Bassey, Efiok, and Webster, 1990b).

Further studies on the respiratory chain of *Vitreoscilla* by Bassey *et al.* revealed that a terminal oxidase was responsible for the sodium pumping (Bassey, Efiok, and Webster, 1990a). *Vitreoscilla* possesses two terminal oxidases, a *bd* type and a *bo* type. As in *E. coli* (Jünemann, 1997) the concentration of *bd* oxidase is likely to increase under conditions of low oxygen (Boerman and Webster, 1982). Both the *bo* and *bd* type oxidases are quinol oxidases (Georgiou and Webster, 1987b; Georgiou and Webster, 1987a). Bassey *et al* initially looked at two possible candidates for the

sodium pump, an NADH dehydrogenase as found in the marine bacteria or the *bo* type oxidase. Although the NADH oxidase activity of the membranes was enhanced more by Na^+ than Li^+ they found that the quinol oxidase activity was only enhanced by Na^+ . When the cytochrome was initially reconstituted into liposomes derived from *E. coli* phospholipids movement of Na^+ both in and out of the liposomes (depending on the Na^+ concentration inside and outside) was detected, and the enzyme catalysed a net uptake of $^{22}\text{Na}^+$ when the liposomes were energised with ascorbate/N,N,N',N'-tetramethyl-1,4-phenylenediamine (TMPD), thus adding to the evidence that the terminal oxidase from *Vitreoscilla* is responsible for pumping sodium (Bassey, Efiok, and Webster, 1990a). Further studies by Park *et al* in which the enzyme was incorporated into liposomes made from *Vitreoscilla* phospholipids saw an increase in extrusion of Na^+ compared with the *E. coli* liposomes (Park, Moon, Cokic, and Webster, 1996). They found an efficiency of 3.93 Na^+ pumped per O_2 consumed when ascorbate/TMPD was used as the substrate

In 1991 Bassey *et al* looked at the synthesis of ATP by *Vitreoscilla* and reported the use of a sodium gradient to generate ATP (Bassey, Efiok, and Webster, 1992). They showed that the amount of ATP generated was dependent on the magnitude of the Na^+ gradient imposed and that anaerobic cells which had been equilibrated with Na^+ were able to synthesise sufficient ATP to double the intracellular concentration when they were exposed to O_2 . From this evidence Bassey *et al* reported that under normal growth conditions Na^+ is probably the main coupling ion for ATP synthesis in *Vitreoscilla*.

All the sodium pumps so far identified have shown a requirement for sodium for maximum activity. Tokuda and Unemoto looked at the sodium pumping NADH oxidase from *Vibrio alginolyticus* and found that at alkaline pH sodium stimulated the generation of membrane potential and rates of oxygen consumption (Tokuda and Unemoto, 1982). Other studies of the sodium pumping NADH oxidase from *Vibrio alginolyticus* and *Klebsiella pneumoniae* have shown that the enzymes require sodium for maximum activity (Tokuda and Unemoto, 1984; Dibrov, Kostyrko et al., 1986; Dibrov, Lazarova et al., 1989; Dimroth and Thomer, 1989). Like other sodium pumping enzymes the sodium pumping terminal oxidase from *Vitreoscilla* has been shown to require sodium for maximum activity (Bassey, Efiok, and Webster, 1990a; Bassey, Efiok, and Webster, 1992; Efiok and Webster, 1990b; Park, Moon, Cokic, and Webster, 1996). The requirement of sodium pumping enzymes for sodium has been used to screen bacteria for sodium pumps. Oh *et al* looked at the correlation between possession of a sodium pump with requirement of sodium for growth in marine bacteria. They found that all the bacteria they tested that failed to grow without sodium possessed a sodium pump and that all the bacteria that had no primary sodium pump grew without sodium. Therefore when screening for possession of a sodium pump a good starting point is the organisms requirement for sodium in the growth media.

One of the aims of the project was to independently verify the sodium pumping action of the cytochrome *bo* from *Vitreoscilla*. Therefore an initial objective was to demonstrate that the activity of the cytochrome *bo* from *Vitreoscilla* could be stimulated by the addition of sodium. Unless sodium dependence could be shown there would be little point in looking for the sodium pump directly. In their 1990 paper

Webster and Bassey showed an increase in activity of both membrane bound and purified enzyme in the presence of sodium chloride. They also showed that this was not an ionic strength effect as lithium did not produce a similar enhancement in activity (Bassey, Efiok, and Webster, 1990a).

5.2 Materials and methods

5.2.1 Reagents

All reagents were purchased from Sigma (Poole, Dorset) unless otherwise stated.

5.2.2 Choice of quinol

In their investigations into the sodium dependent nature of Cytochrome *bo* from *Vitreoscilla*, Webster's group used menadiol as the quinol substrate (Bassey, Efiok, and Webster, 1990a; Bassey, Efiok, and Webster, 1990b; Bassey, Efiok, and Webster, 1992; Efiok and Webster, 1990a; Efiok and Webster, 1992; Kim, Chi et al., 2000; Park, Moon, Cokic, and Webster, 1996; Webster, Park et al., 1995; Park, Moon, Cokic, and Webster, 1996).

5.2.3 Preparation of quinols from quinones

Quinols were produced from appropriate quinone as follows (Rich, 1981). All solutions and air spaces were flushed with oxygen free nitrogen (boc Guildford, Surrey) prior to use. The quinone (0.25 g) was dissolved in 50 ml of diethyl ether in a separating funnel. An equal volume of dithionite buffer was added (1 M potassium phosphate pH 7 containing 1 g of sodium dithionite). The funnel was shaken vigorously and the dithionite buffer layer poured off. The ethereal layer was then shaken with a second volume of dithionite buffer to ensure complete reduction. The dithionite buffer layer was poured off and the ethereal layer was passed through 30 g of anhydrous sodium sulphate in a sintered glass funnel to remove any remaining water. The diethyl ether was then driven off in a rotary evaporator (with the exception

of menadiol). To prevent reoxidation the diethyl ether was driven off the menadiol using nitrogen blown across the solution. Quinols were stored at room temperature in the dark. Solutions were made up in 96% ethanol containing 10 mM HCl and stored at -20 °C. Concentration of stock decylubiquinol was determined using the extinction coefficient $8 \text{ mM}^{-1}\text{cm}^{-1} \Delta_{272(\text{oxidised})-247(\text{reduced})}$ (Zheng, Shoffner et al., 1990) and was found to be 26 mM.

5.2.4 Isolation of respiratory membranes and purification of enzyme

Respiratory membranes were prepared and cytochrome *bo* isolated from them as described in Chapter 3 Page 75.

5.2.5 Measurement of oxidase activity

Quinone undergoes a $2\text{H}^+ + 2\text{e}^-$ reduction to form ubiquinol. The reduced and oxidised forms of ubiquinol have different absorption spectra and so can be distinguished spectroscopically. It is therefore possible to monitor the appearance of ubiquinone due to the action of a quinol oxidase. The absorbance spectra of the reduced and oxidised quinol were compared and a wavelength was chosen where there was a difference in absorbance between the two spectra. The absorbance at this wavelength was then monitored during ubiquinol oxidase reactions to give an indication of the appearance of the oxidised form of the substrate.

5.2.6 Assay procedure

Each assay was performed in a 1 ml quartz cuvette. The total assay volume was 500 μl . Buffer B (see appendix A Page 181) was added to the cuvette followed by the

desired volume of sodium, lithium or potassium chloride solution. The membranes were added and the reaction was started by the addition of quinol. The reaction was monitored at the appropriate wavelength for 3 min (see Results Page 135).

5.2.7 Spectroscopy

All measurements of enzyme reactions were carried out in a Perkin Elmer Lambda bio 20 dual beam spectrophotometer (Wellesley, Massachusetts), fitted with a circulating water bath set at 25 °C. The resulting time courses of quinol oxidation were exported into Sigma Plot and appropriate curves were fitted. The initial rate was calculated by dividing the span times the rate constant (from the fitted curve) with the appropriate extinction coefficient giving the initial rate in mMs^{-1} . The resulting rate was multiplied by 1000 to give μMs^{-1} and divided by 2 to take into account the volume in the cuvette (500 μl) giving the rate in $\text{nmol ml}^{-1}\text{s}^{-1}$. An extinction coefficient for menadiol could not be found and so concentration is quoted as volume of stock added.

5.3 Results

5.3.1 Choice of wavelength and quantification of quinol stock solutions

The wavelengths used for activity measurements and for the measurement of quinol concentration were determined spectroscopically using *Vitreoscilla* membranes to oxidise the reduced quinol. Figure 5.1 Page 136 shows the reduced and oxidised spectrums of decylubiquinol. The wavelength chosen for the activity measurements using decylubiquinol was 278 nm at this wavelength there was a good difference between the two spectra. For duroquinol a wavelength of 288 nm was used and for menadiol 262 nm was used. The concentration of decylubiquinol was determined using the appropriate extinction coefficient.

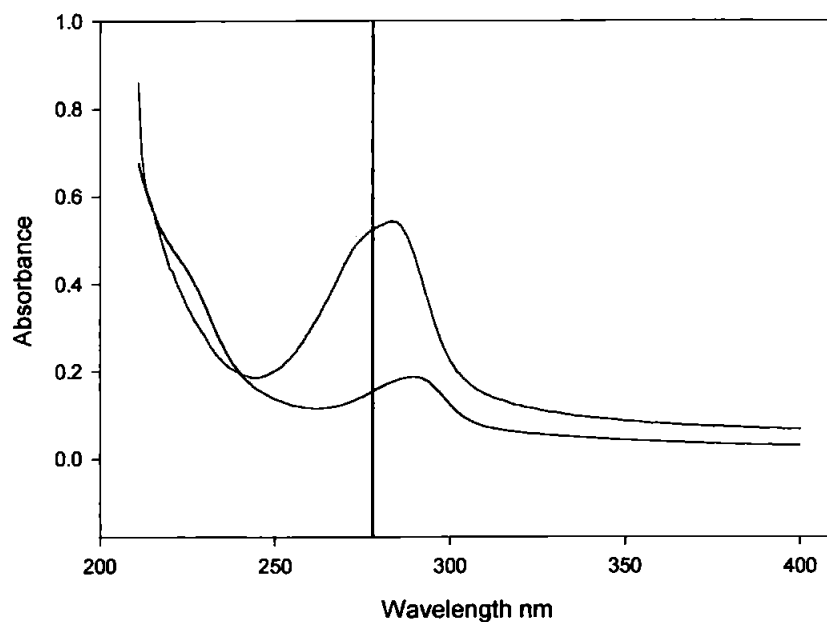


Figure 5.1 Reduced and oxidised decylubiquinol (— reduced, — oxidised). Decylubiquinol was produced as described in the materials and methods section. 0.5 μl of stock decylubiquinol was added to 500 μl of buffer and the absorbance was measured from 200 to 400 nm. The decylubiquinol was oxidised with the addition of 1 μl of $84\mu\text{g l}^{-1}$ membranes (measured by protein content) from *Vitreoscilla* the absorbance between 200 and 400 nm was measured every 23 seconds until oxidation was complete.

5.3.2 The oxidation of duroquinol by respiratory membranes from *Vitreoscilla* strain LB13

The first quinol tested was duroquinol. The quinol was prepared from duroquinone and assays carried out as described on Page 132. The wavelength used to monitor duroquinol oxidation was 288 nm. Assays with duroquinol were repeated three times. Figure 5.2 Page 138 shows the resulting plot of initial rate of quinol oxidation versus increasing duroquinol concentration. It can clearly be seen that above 160 μM the measurements of activity of the membranes became variable and it was not possible to accurately saturate the enzyme.

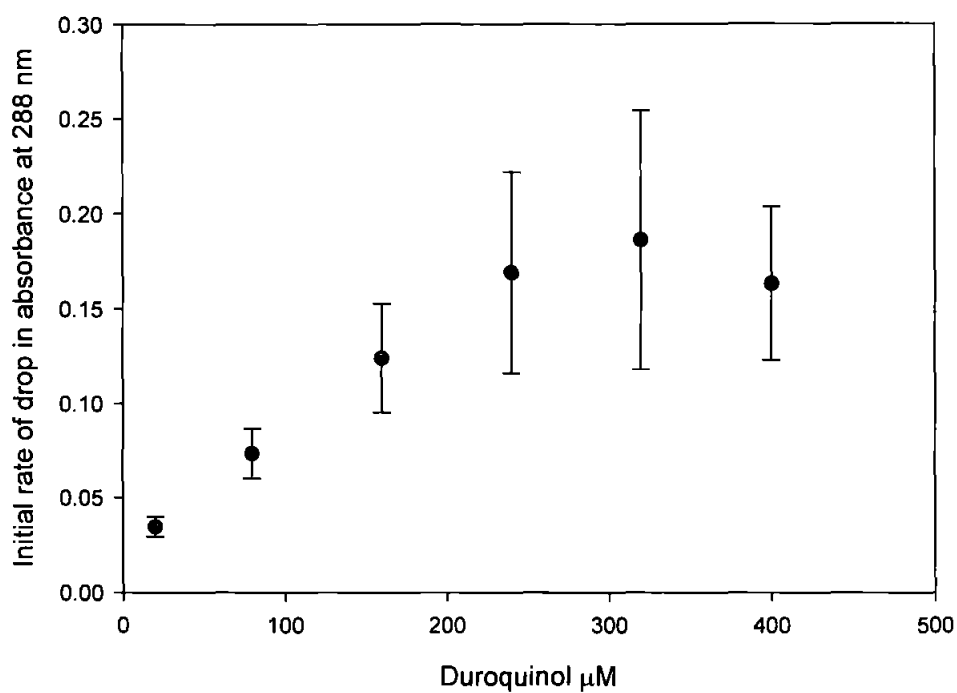


Figure 5.2 The effect of substrate concentration on the rate of oxidation of duroquinol by respiratory membranes from *Vitreoscilla* LB13. Final volume of 0.5ml contained 22.5 mM potassium phosphate buffer pH 7.6, 50 μl of 4.2g l^{-1} membranes (measured by protein content). Oxidation was measured at 288 nm for 3 minutes.

5.3.3 The oxidation of decylubiquinol by respiratory membranes from *Vitreoscilla* strain LB13

The rates of activity at higher levels of duroquinol were found to be variable and so a different quinol was tried. Decylubiquinol was produced as described in the Materials and Methods section Page 132. Figure 5.2 (Page 138) shows the initial rate of oxidation for each concentration of decylubiquinol. Unlike the duroquinol the quinol oxidation activity was successfully saturated by the higher concentrations. From the data in Figure 5.3 the K_m of the quinol oxidase activity for this substrate was estimated. The K_m and V_{max} were calculated by fitting a rectangular hyperbola to the data and were found to be $9.8 \mu\text{M}$ and 40.5 nmol s^{-1} , respectively.

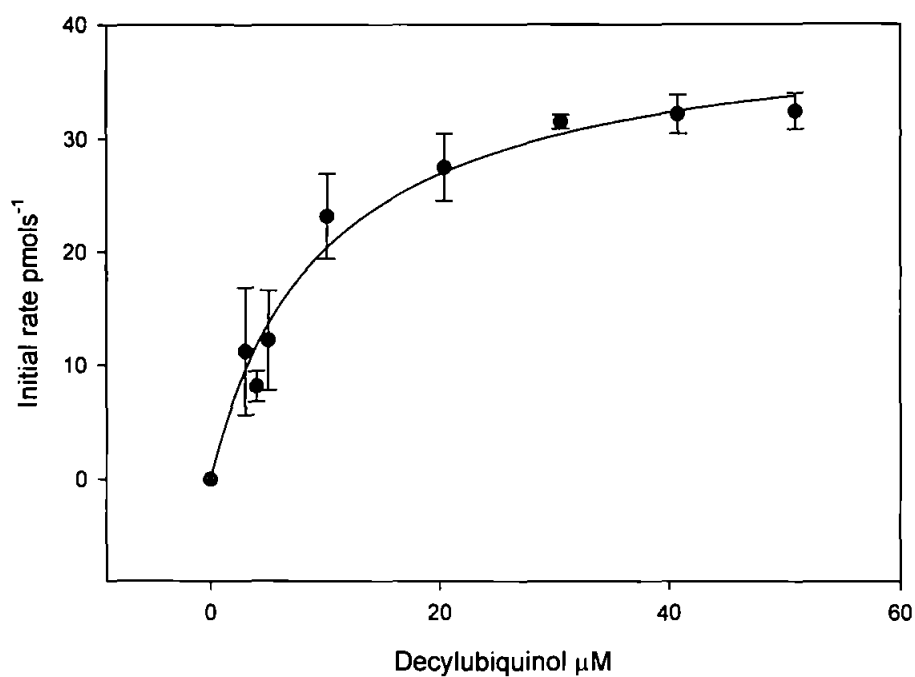


Figure 5.3 The effect of substrate concentration on the rate of oxidation of decylubiquinol by respiratory membranes from *Vitreoscilla* LB13. Each point represents the average of three replicates \pm 1 standard deviation. Final volume of 0.5ml contained 22.5mM potassium phosphate pH 7.6, 5 μ l of 4.2g l⁻¹ membranes (measured by protein content). Oxidation was measured at 278 nm for 3 minutes.

5.3.4 The effect of sodium, lithium and potassium chlorides on the rate of quinol oxidation by *Vitreoscilla* LB13 respiratory membranes

The quinol oxidase activity in *Vitreoscilla* LB13 membranes was tested for sensitivity to sodium, lithium and potassium ions. The experiment was conducted as above (at substrate concentrations either equivalent to the K_m , or sufficient to nearly saturate the enzyme, i.e. ten times K_m) but between 0 and 500 mM of each ion was added to the buffer prior to addition of the membranes. Figure 5.3 and 5.4 (Pages 140 and 142, respectively) show the % quinol oxidase activity of the membranes (relative to the control) against ion concentration at $0.5 V_{max}$ and V_{max} respectively. At both $0.5 V_{max}$ and V_{max} the addition sodium chloride caused a similar decrease in activity, this trend continued as the sodium ion concentration was increased and 500 mM sodium chloride caused a reduction in activity of around 54 %. At no point was a stimulation of activity observed. The addition of lithium and potassium ions also caused a drop in activity but to differing extents.

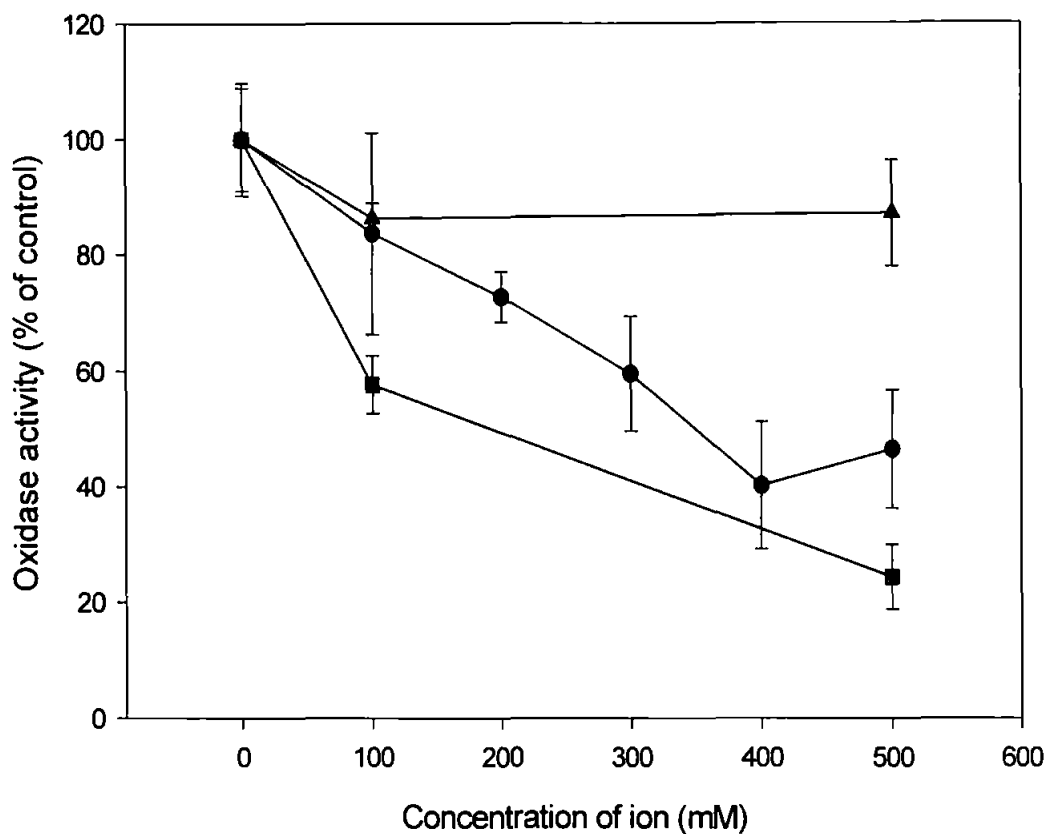


Figure 5.4 The effect of sodium chloride (●), potassium chloride (▲) and lithium chloride (■) on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain LB13 at 0.5 V_{max} of the enzyme activity. Final volume of 0.5ml contained between 0 and 500mM sodium, potassium or lithium chloride, 5 μ l of 4.2g l^{-1} membranes (measured by protein content), 9.8 μ M decylubiquinol, volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes. Each point represents the average of three replicates, \pm 1 standard error.

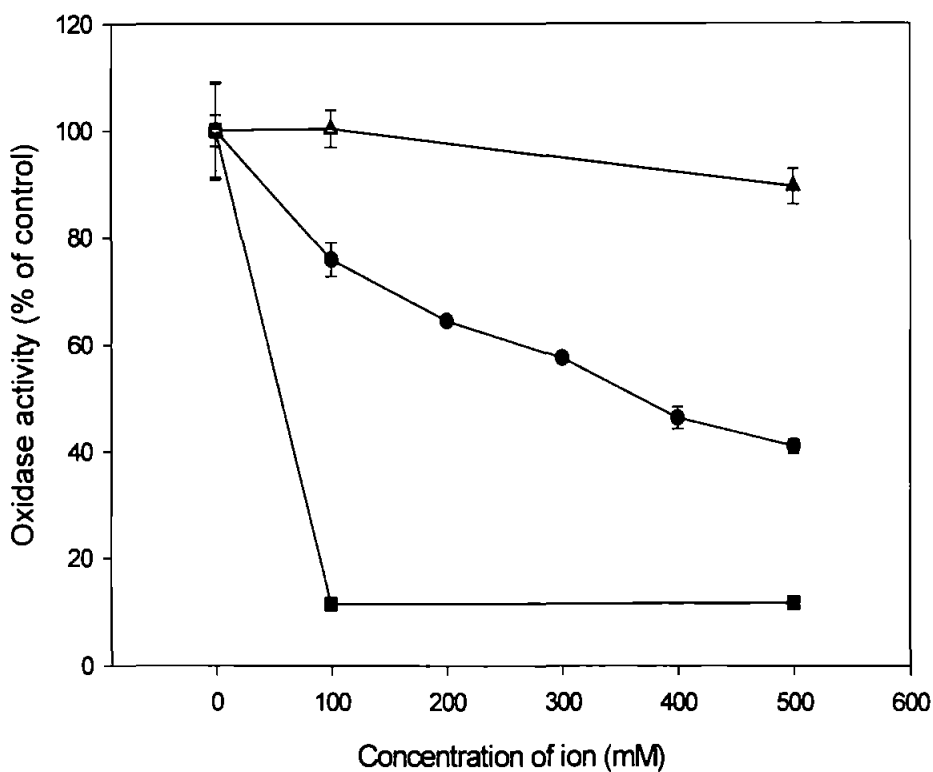


Figure 5.5 The effect of sodium chloride (●), potassium chloride (▲) and lithium chloride (■) on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain LB13 close to the V_{max} of the enzyme. Final volume of 0.5ml contained between 0 and 500mM sodium, potassium or lithium chloride, 5 μ l of 4.2g l^{-1} membranes (measured by protein content), 51 μ M decylubiquinol, volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes. Each point represents the average of three replicates, \pm 1 standard error.

5.3.5 The effect of sodium chloride and monensin and/or FCCP on the rate of decylubiquinol oxidation by *Vitreoscilla* LB13 respiratory membranes

The quinol oxidase activities measured in the experiments in the previous sections were obtained using enzyme that was contained in respiratory membranes. It is assumed that these membranes had spontaneously formed vesicles in aqueous suspension. As the enzyme was operating in a closed system it is possible that the activity of the enzyme was affected by build up of membrane potential (and to a lesser extent ion gradient). The build up of membrane potential across the vesicle membrane could have resulted in a reduction of activity of the enzyme (if it is a sodium pump) particularly if the enzyme were predominantly right oriented i.e. the same orientation as is found in the bacterium. It is also possible that the enzyme would be insensitive to sodium ion if the dependent part of the enzyme were facing inwards. For these reasons the sodium activity experiments were repeated with the addition of monensin, a Na^+ specific ionophore that catalyses Na^+/H^+ exchange across membranes (Sandeaux, Sandeaux et al., 1982), and carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), a protonophore.

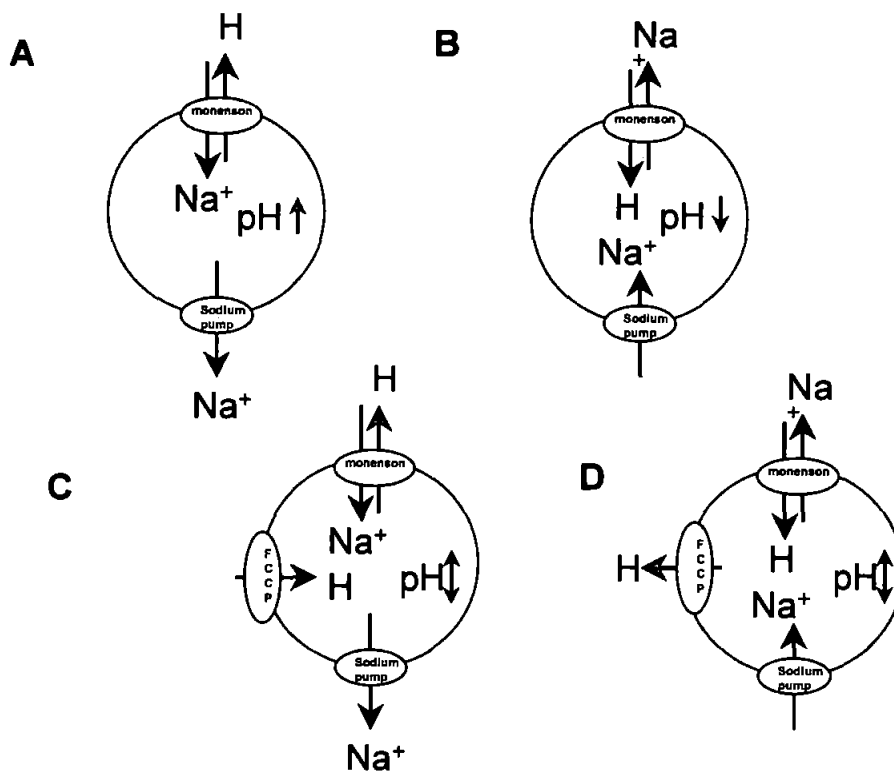


Figure 5.6 The proposed effect of ionophores on the respiratory membrane vesicles used in activity experiments. Monensin would allow sodium ions to pass through the membranes in exchange for hydrogen ions. A rise or drop in pH (depending on orientation of the enzyme) is predicted in the vesicles when treated with monensin alone. An H^+ ionophore would prevent a build up of membrane potential and hence prevent respiratory control from reducing the pumping of sodium ions by the enzyme. The addition of FCCP allows the flow of hydrogen ions to equilibrate over the membrane and prevents a build up of membrane potential.

The monensin would allow sodium ions to pass through the membranes in exchange for hydrogen ions. As can be seen in Figure 5.6 panel A and B (Page 145) a rise or drop in pH (depending on orientation of the enzyme) is predicted in the vesicles when treated with monensin. An H^+ ionophore was added to the reaction to exchange H^+ ions and prevent a build up of membrane potential and hence prevent respiratory control from reducing the pumping of sodium ions by the enzyme. As can be seen in Figure 5.6 panel C and D (Page 145) the addition of FCCP allows the flow of hydrogen ions to equilibrate over the membrane and prevents a build up of membrane potential. This combination of ionophores would cause the sodium pump to be released from respiratory control. The experiment was conducted as described for the sodium chloride experiment with the exception that either 10 μ l of water or 10 μ l of monensin, and/or 10 μ M FCCP (both at 10 μ M) or 10 μ l of ethanol were added to the buffer prior to the addition of the membranes.

Figure 5.6 (Page 145) shows the effect of both ionophores on the effect of sodium chloride on the rate of quinol oxidation by respiratory membranes. No difference was observed between the test activities and the control. The rate of oxidation fell as the sodium chloride concentration increased. Experiments conducted using either monensin or FCCP also showed no difference in the effect of sodium chloride on the activity of the enzyme.

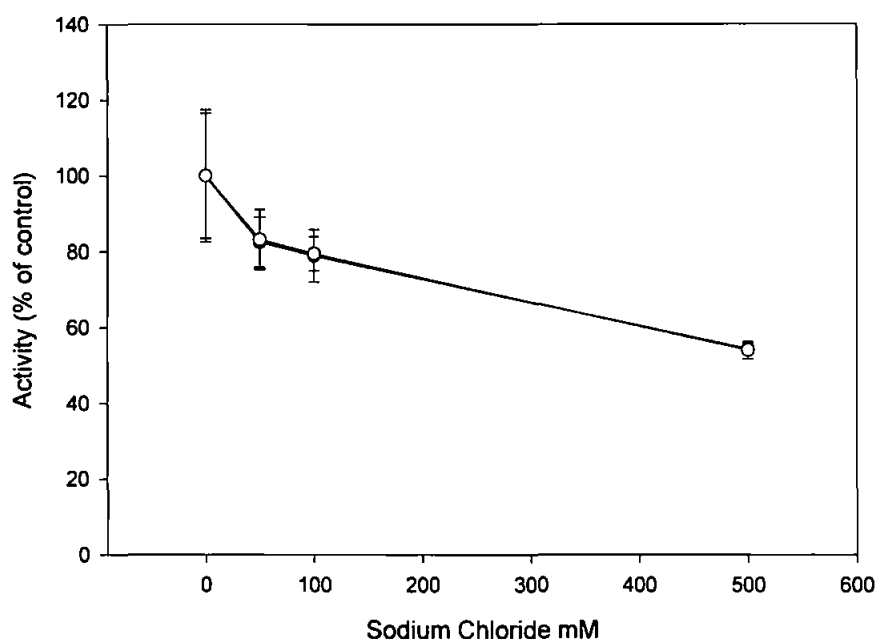


Figure 5.7 The effect of sodium chloride, FCCP and monensin on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain LB13 approaching the calculated V_{max} of the enzyme. ● Without FCCP or monensin ○ With 0.2μM FCCP and monensin 0.2μM. Final volume of 0.5ml contained between 0 and 500mM sodium chloride, 5 μl of 4.2g l^{-1} membranes (measured by protein content), 51 μM decylubiquinol, 10 μl of 10 μM FCCP and 10μl of 10 μM monensin. The volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes. Each point represents the average of three replicates, \pm 1 standard error.

5.3.6 The effect of substrate concentration on the rate of oxidation of menadiol by respiratory membranes from *Vitreoscilla* LB13

As none of the activity experiments with decylubiquinol showed enhancement with sodium chloride another quinol was tested. Menadiol was used because it is the quinol used by Webster and co-workers in their activity experiments (Bassey, Efiok, and Webster, 1990a). Menadiol proved more difficult than either duroquinol or decylubiquinol to produce. The procedure was followed as previously described (Page 132). The first production of menadiol turned purple when being evaporated to dryness, a second batch was produced but this time the quinol was dried using oxygen free nitrogen (Boc, Guildford, Surrey). The resulting menadiol powder was dissolved in 5 ml of acidified ethanol and stored at -20°C . The stock solution was diluted 10 fold in acidified ethanol before use.

The enzyme was not successfully saturated with this substrate and therefore the K_m and V_{max} were not calculated; nevertheless the substrate was used for further activity experiments.

5.3.7 The effect of sodium chloride on the rate of oxidation of menadiol by respiratory membranes from *Vitreoscilla* LB13

The sodium chloride experiment was repeated using menadiol as the substrate. The experiment was conducted as before with the exception that 5 μ l of menadiol (as prepared) was used to start the reaction that was monitored at 262 nm for 3 minutes. Figure 5.8 Page 150 shows the activity against concentration of sodium chloride. At 100 mM sodium chloride a drop in activity compared to the control of 20 % was observed. The activity continued to drop as the concentration of sodium chloride increased reaching 53 % of the control at 500 mM sodium chloride.

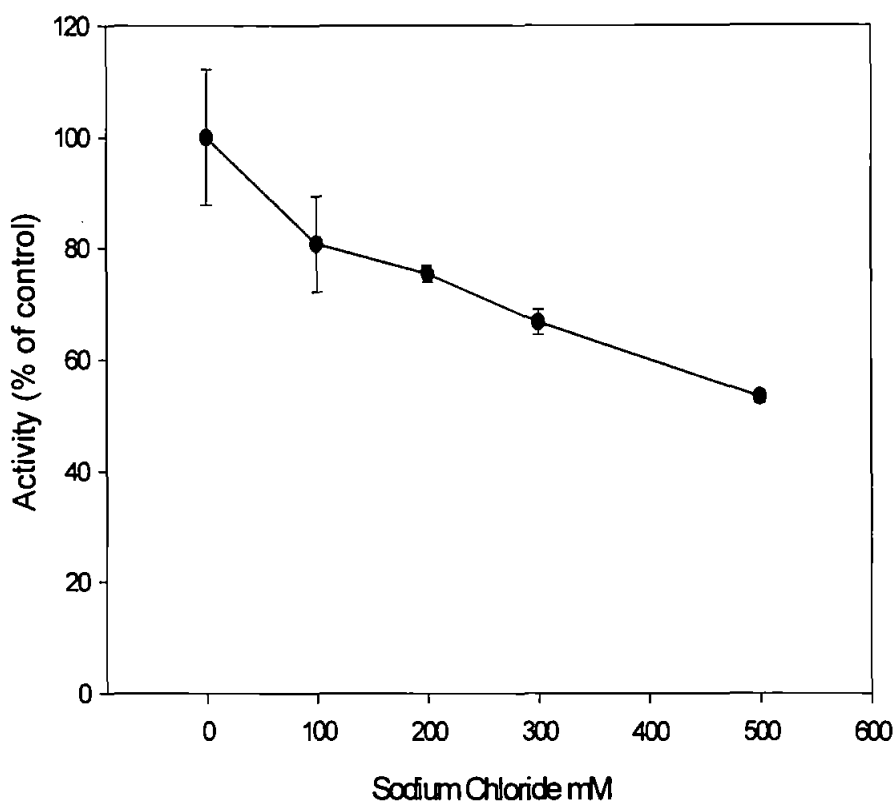


Figure 5.8 The effect of sodium chloride on the rate of oxidation of menadiol by respiratory membranes from *Vitreoscilla* strain LB13. Final volume of 0.5ml contained between 0 and 500mM sodium chloride, 5 μ l of 4.2g l⁻¹ membranes (measured by protein content), 5 μ l of menadiol (as prepared) volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 262 nm at 27°C for 3 minutes, \pm 1 standard error.

5.3.8 The effect of substrate concentration on the rate of oxidation of decylubiquinol by respiratory membranes from *Vitreoscilla* C1

At this point a new strain of *Vitreoscilla* was obtained from Dr. Dale Webster. This strain, C1, was claimed to be the strain from which the sodium-pumping *bo*-type oxidase had previously been isolated and characterised, and so it was tested for sodium sensitivity in the same way strain LB13 had been tested. Decylubiquinol was tested first and the effect of the concentration of this substrate on the oxidation activity of the respiratory membranes was examined. Membranes were isolated as described in Chapter 3 Page 75. The experiment was conducted as described for LB13 respiratory membranes. The resulting data were treated as described for LB13 membranes. Figure 5.9 Page 152 shows the initial rate for each concentration of decylubiquinol the fitted curve is not a good fit for the data indicating that more than one oxidase is acting on the substrate. From this plot the K_m and V_{max} for this substrate were calculated and were found to be $12.3 \mu M$ and 152 nmol s^{-1} , respectively. Further experiments were conducted using the concentration of substrate that gave $0.5 V_{max}$. When related to the total protein present the V_{max} of *Vitreoscilla* strain C1 is $13.33 \text{ nmols}^{-1}\text{mg}^{-1}$. This compares to only $0.96 \text{ nmols}^{-1}\text{mg}^{-1}$ for *Vitreoscilla* strain LB13. The c1 strain has a far higher activity than LB13.

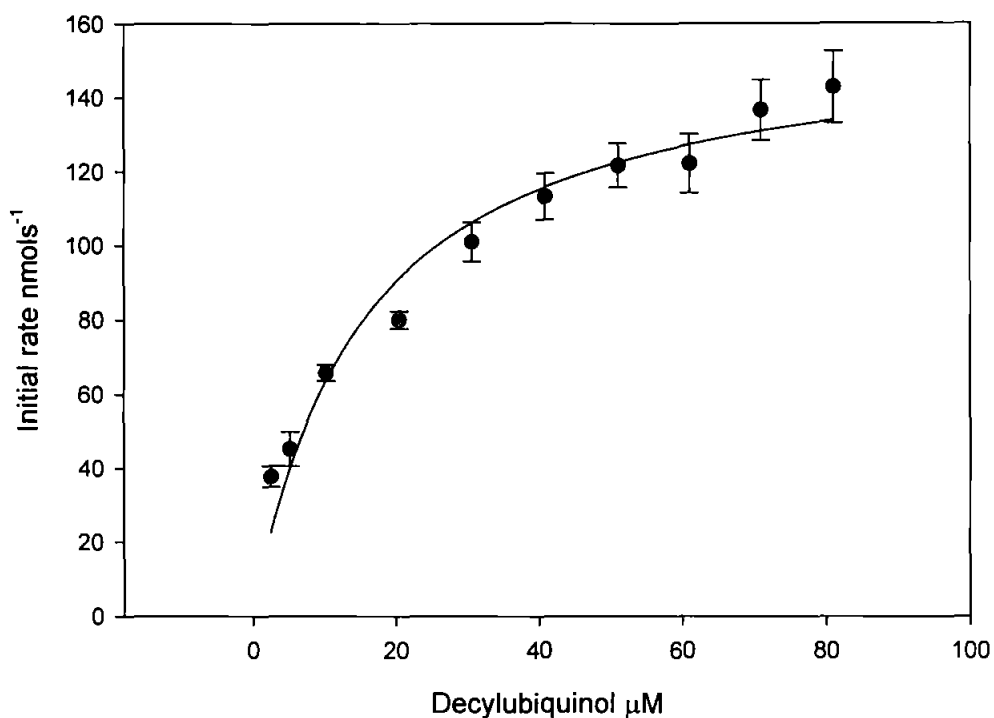


Figure 5.9 The effect of substrate concentration on the rate of oxidation of decylubiquinol by respiratory membranes from *Vitreoscilla* C1. Each point represents the average of three replicates ± 1 SEM. Final volume of 0.5ml contained 22.5mM potassium phosphate pH 7.6, 5 μl of 1.14 g l^{-1} membranes (measured by protein content). Oxidation was measured at 278 nm for 3 minutes.

5.3.9 The effect of sodium chloride on the rate of oxidation of decylubiquinol and menadiol by respiratory membranes from *Vitreoscilla* strain C1.

Once the K_m for decylubiquinol had been established the oxidation of decylubiquinol and menadiol was tested for sensitivity to sodium chloride. The experiment was conducted as described for LB13 membranes with the exception that 12.3 μ M decylubiquinol or 5 μ l of menadiol (1 in 10 dilution of stock) was added to start the reaction. Figure 5.10 Page 154 shows the activity (as percentage of the control activity) of the membranes at each sodium chloride concentration for both substrates. Using decylubiquinol, at 500 mM sodium chloride there was a reduction in activity of 81 %. The oxidation of menadiol was also inhibited by the addition of sodium chloride with 500 mM causing an inhibition of 39 %.

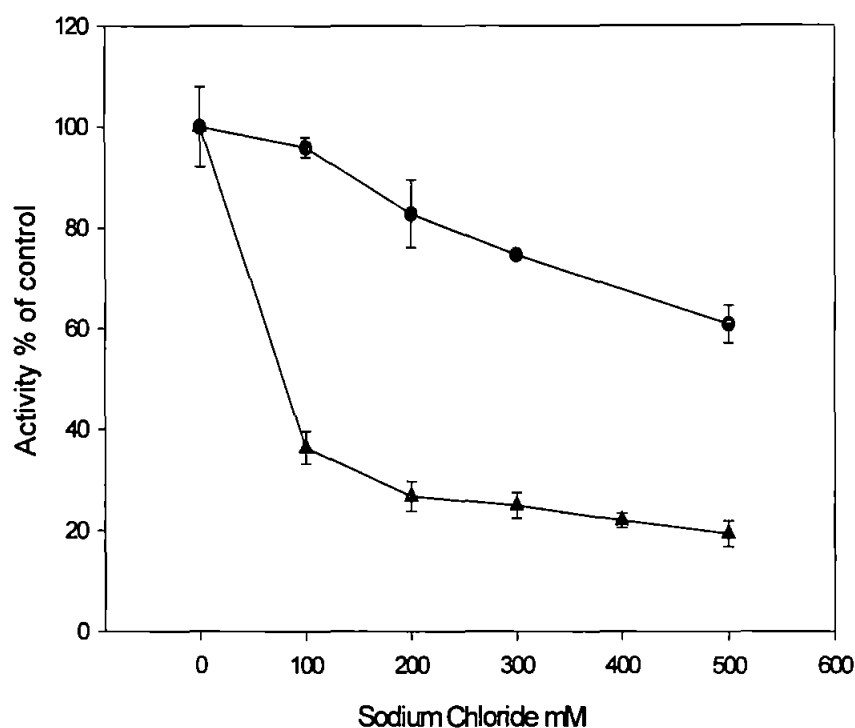


Figure 5.10 The effect of sodium chloride on the rate of decylubiquinol (▲) and menadiol (●) oxidation by respiratory membranes from *Vitreoscilla* strain C1 at $0.5 V_{max}$ of the enzyme. Final volume of 0.5ml contained between 0 and 500mM sodium chloride, 5 μ l of 1.14 g l^{-1} (measured by protein content), 12.3 μ M decylubiquinol or 5 μ l of menadiol (1 in 10 dilution) volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes. Each point represents the average of three replicates, ± 1 standard error.

5.3.10 The effect of sodium chloride, monensin and FCCP on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain C1

As with the LB13 membranes the possibility existed that the C1 membranes had formed vesicles with the enzyme predominantly right oriented. In order to ensure the release of respiratory control, the activity of the enzyme was measured at increasing sodium chloride concentrations in the presence of monensin and FCCP. The experiment was conducted as described previously with the exception that either 0.2 μ M monensin and 0.2 μ M FCCP or 10 μ l of water and 10 μ l of ethanol were added prior to addition of the membranes. Figure 5.11 Page 156 shows the activity of the membranes at increasing sodium chloride concentrations with or without monensin and FCCP. Little difference was observed between the two sets of results with inhibition at 500 mM sodium chloride reaching 88 % with FCCP and monensin and 81 % for the control.

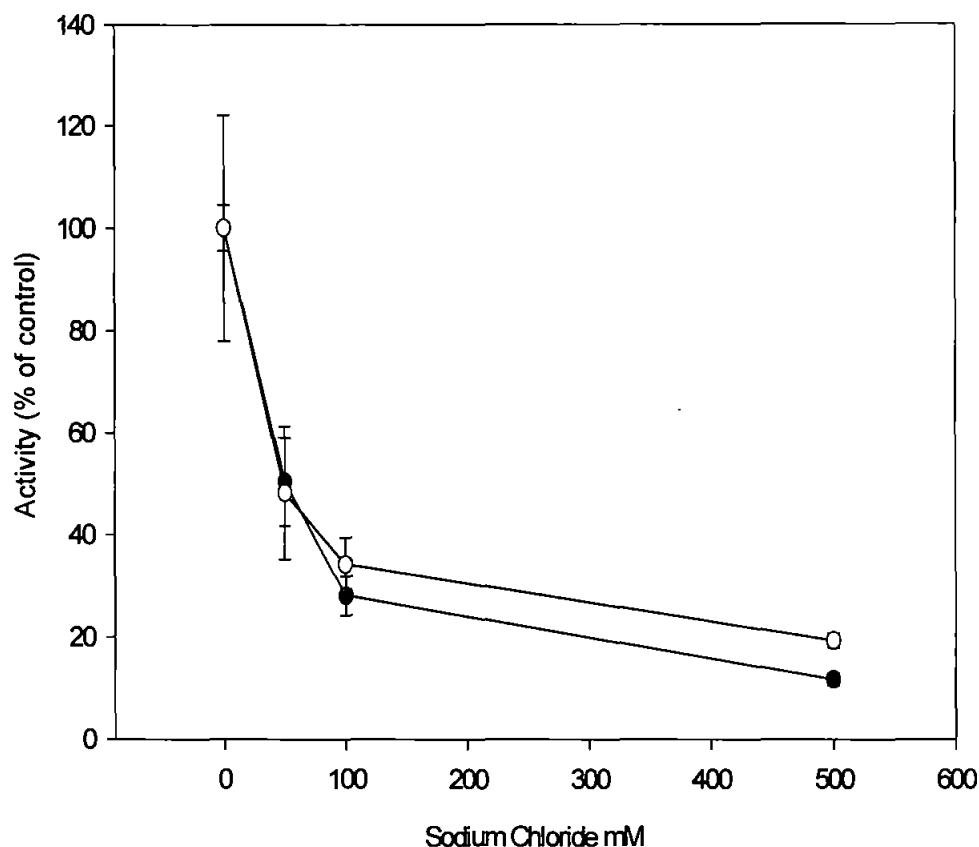


Figure 5.11 The effect of sodium chloride, monensin and FCCP on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain C1 at the calculated K_m of the enzyme. ● With monensin and FCCP ○ Control. Final volume of 0.5ml contained between 0 and 500mM sodium chloride, 5 μ l of 1.14 $g\ l^{-1}$ (measured by protein content), 12.3 μ M decylubiquinol, 0.2 μ M monensin and 0.2 μ M FCCP. The volume was made up to 0.5ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes, \pm 1 standard error.

5.3.11 Inhibition of decylubiquinol oxidation by potassium cyanide

Respiratory membranes had been used for all the previous measurements of activity. As *Vitreoscilla* produces two terminal oxidases (a *bo* type and a *bd* type) it was necessary to determine what proportion of the activity measured so far was caused by *bd* contained in the membranes. The *bd* in the membranes could have been masking any effect the sodium ions were having on the *bo*. The activity experiments were therefore repeated with extracted enzyme, that is enzyme that had been treated with octylglucoside, a detergent that *bd* from *E. coli* does not function in (Lorence, Miller et al., 1984). To ensure that *bd* in these samples was either absent or inhibited the oxidation was monitored in the presence of potassium cyanide. Cytochrome *bd* is relatively insensitive to KCN whilst cytochrome *bo* is not (Pudek and Bragg, 1974). Samples of extracted enzyme, membranes and purified *bo* were tested for their sensitivity to KCN.

Table 5.1 Inhibition of cytochrome *bo/bd* with KCN. Membranes, extracted membranes or purified enzyme were added to 1 ml of potassium phosphate buffer with either 200 μ l of 100 mM KCN or 200 μ l water. The reaction was started by the addition of 5 μ l of decylubiquinol (1 in 10 dilution of stock) and oxidation was monitored at 278 nm for 3 minutes. Each figure represents the average of three replicates \pm 1 Standard deviation.

State of enzyme	% inhibition by KCN
Contained in membrane	53% \pm 6.74
Treated with octylglucoside	No detectable activity
Purified	No detectable activity

Table 5.1 shows the % inhibition for each sample compared to the control. The whole membranes showed 53 % inhibition by KCN while both the octylglucoside treated membranes and the purified enzyme were inhibited to the point that no activity was detectable.

5.3.12 The effects of sodium, potassium and lithium chlorides on the rate of decylubiquinol and menadiol oxidation by respiratory membranes from *Vitreoscilla* LB13 treated with octylglucoside.

Some of the activity experiments were repeated with membranes that had been treated with octylglucoside to determine the contribution that cytochrome *bd* had made to the previous activity measurements. The first experiments were conducted as described previously with the exception that treated membranes were used in place of whole membranes. Table 5.1 Page 158 shows the resulting activity measurements for each ion at 500 mM. Inhibition of the activity was observed in the presence of sodium and lithium chloride whilst potassium chloride had little effect.

Table 5.2 The effect of sodium lithium and potassium chloride on the rate of decylubiquinol oxidation by respiratory membranes from *Vitreoscilla* strain LB13 treated with octylglucoside at 0.5 V_{max} of the enzyme. Final volume of 1ml contained between 0 and 500mM sodium, lithium or potassium chloride (results for 500 mM shown), 0.002 μ M *bo* (measured by difference spectroscopy), 9 μ M of decylubiquinol. The volume was made up to 1ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 278 nm at 27°C for 3 minutes. Each percentage represents the average of three replicates, +/- 1 standard error.

Ion (500 mM)	Activity % of control \pm 1 SEM n=3	
	Treated	Untreated
Sodium	57 \pm 1	46 \pm 10
Lithium	43 \pm 12	24 \pm 6
Potassium	99 \pm 7	87 \pm 9

The treated membranes were then tested with menadiol and sodium chloride. The experiment was conducted as previously described with the exception that treated membranes were added in place of whole membranes. Figure 5.12 Page 162 shows the activity of the membranes at increasing sodium chloride concentrations. Addition of 100 mM sodium chloride caused a reduction in activity of 25 %. The activity continued to decrease with the addition of increased concentrations of sodium chloride reaching 52 % at 500 mM.

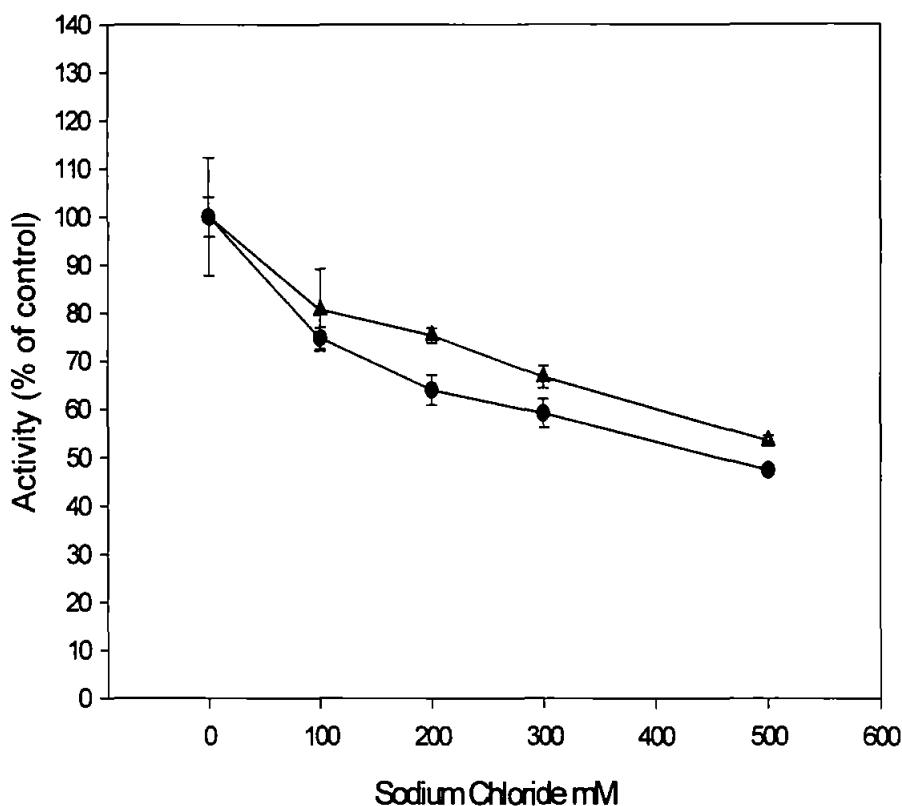


Figure 5.12 The effect of sodium chloride on the rate of menadiol oxidation by respiratory membranes from *Vitreoscilla* strain LB13 treated with octylglucoside. ● Membranes treated with octylglucoside. ▲ Untreated membranes. Final volume of 1ml contained between 0 and 500mM sodium chloride, 0.002 μ M *bo* (measured by difference spectroscopy), 0.5 μ l of menadiol (as prepared). The volume was made up to 1ml with potassium phosphate buffer pH 7.6. Oxidation was monitored at 262 nm at 27°C for 3 minutes. Each point represents the average of three replicates, \pm 1 standard error.

5.4 Discussion

At the outset of the project it was important to establish whether the activity of cytochrome *bo* could be enhanced by the addition of sodium chloride. Webster and his group have shown that activity of the enzyme is increased by the addition of sodium chloride and that lithium and potassium cannot substitute (Bassey, Efiok, and Webster, 1990b; Bassey, Efiok, and Webster, 1990a). Although two different quinols and two different strains of *Vitreoscilla* were tried, stimulation of quinol oxidase activity by sodium ion was not observed; in fact an inhibition of activity by both sodium and lithium ions was noted.

As it was not thought that choice of substrate would affect the activity of the enzyme the first quinol tested was duroquinol not menadiol as Webster et al had used. The activity measurements with this substrate became variable at high substrate concentrations and so decylquinol was tried. It was possible to saturate the enzyme with this substrate, allowing the calculation of the K_m of the enzyme (within membranes) from both LB13 and C1 (the strain reported to possess a sodium pumping terminal oxidase by Webster's group). Activity measurements using LB13 membranes with this substrate showed that both lithium chloride and sodium chloride inhibited activity at both $0.5 V_{max}$ and close to V_{max} whilst potassium chloride had little effect. A greater inhibition of activity by sodium chloride was seen in the C1 respiratory membranes. The explanation for the lack of effect of potassium on the LB13 membranes could lie in the fact that potassium was used in the buffer during the activity measurements and so the enzyme was already exposed to this ion. Lithium chloride proved to be more inhibitory than sodium chloride. There was also a difference in the effect of lithium at V_{max} and $0.5 V_{max}$. The maximum inhibition of

oxidation activity occurred at a lower concentration of lithium at V_{\max} than at $0.5 V_{\max}$ indicating that the K_m of the enzyme (or enzymes) had decreased, i.e. that the affinity of the enzyme for the substrate had increased. The inhibition of the enzyme activity by sodium chloride was similar for both the V_{\max} and $0.5 V_{\max}$ indicating that the effect was on the V_{\max} of the enzyme (or enzymes). In later experiments using membranes treated with octylglucoside to inhibit the *bd* component of the membranes lithium and sodium chloride had a similar inhibitory effect, suggesting that the difference in the first experiments was due to an effect on *bd* rather than *bo*.

Enhancement of activity with sodium chloride had clearly not been shown with the initial activity experiments with either strain of *Vitreoscilla*. Therefore possible explanations for the lack of enhancement were investigated. The enzyme used in the initial experiments was contained in respiratory membranes that had probably formed vesicles. The possibility that the enzyme was being affected by respiratory control due to a build up of ions and/or charge on one side of the vesicles needed to be investigated and so the activity measurements were repeated on membranes from both LB13 and C1 and ionophores FCCP and monensin were added. Even with ionophores present there was still no enhancement of activity with sodium chloride with both the treated and untreated membranes behaving in a similar way. Respiratory control was not therefore found to be affecting the enzyme activity.

With the possibility that the enzyme was being affected by respiratory control ruled out other explanations for the lack of enhancement were investigated. In their activity measurements on cytochrome *bo* from *Vitreoscilla* Bassey et al used menadiol as the substrate (Bassey, Efiok, and Webster, 1990a). An initial attempt to reduce

menadione for use in activity experiments proved unsuccessful and so decylubiquinol was used. When it became clear that the activity of the enzyme was reduced by sodium chloride when using decylubiquinol a second, successful attempt was made to produce menadiol for use in activity experiments. As with the decylubiquinol as substrate sodium chloride caused a reduction in the rate of quinol oxidation in both LB13 and C1 membranes. The level of inhibition was consistent with the results obtained using decylubiquinol; the choice substrate was therefore not thought to be responsible for the lack of enhancement of activity with sodium.

At this stage it was again possible that the activity of *bd* in the membranes was masking any enhancement by sodium chloride. The activity experiments were therefore repeated using membranes from LB13 that had been treated with octylglucoside. Certain detergents inhibit cytochrome *bd* (Lorence, Miller, Borochoy, Faiman-Weinberg, and Gennis, 1984). Membranes treated with octylglucoside showed almost 100 % inhibition by cyanide indicating that after treatment with octylglucoside *bo* was responsible for all the quinol oxidation activity.

Once again decylubiquinol was used as this substrate was the easiest to produce and provided the best saturation curve. No difference was seen between the activity rate of the treated and the untreated membranes. Inhibition by sodium chloride is similar in both cases. The sodium chloride therefore must be inhibiting both enzymes.

To complete the picture menadiol was also tested and again no enhancement of activity was seen. There was no difference between the treated and untreated

membrane activities. As with decylubiquinol the removal of the *bd* activity had little effect on the inhibition by sodium chloride.

Although there was no difference between the results for potassium and sodium the lithium did show a difference. The inhibition of the untreated membranes with lithium was almost twice that of the treated. The treated membranes showed a similar inhibition to the sodium treated and untreated membranes. Lithium ions therefore must be having an inhibitory effect on the *bd* component of the membranes. It is unlikely that the lithium is having an effect on the quinol binding site of *bd*. The site is in the hydrophobic part of the membrane and would not be accessible to lithium. It has also been suggested that cytochrome *bd*'s resistance to ionic haem ligands may be caused by a hydrophobic environment at the oxygen binding site (Jünemann, 1997), making this site an unlikely target for inhibition by lithium. The effect is probably more general, the lithium may interfere with some aspect of proton uptake by the enzyme.

The activity experiments show that the terminal oxidase from *Vitreoscilla* is not sodium dependent and is therefore not likely to be a sodium pump. Evidence from bacteria that do possess sodium pumps suggests that very specific environmental conditions favour the use of a sodium pump over a hydrogen pump. The question remains; does *Vitreoscilla* live in conditions where this would be true? Padan and his colleagues found that bacteria living in alkaline conditions had an intracellular pH that is lower than the surrounding pH (Padan, Zilberstein et al., 1976). Pumping H^+ from the cytoplasm into a higher pH results in the generation of a $\Delta\psi$ which is counterbalanced by the ΔpH of the opposite direction. Therefore, $\Delta\mu_{H^+}$ is too low to

sustain functions of the cytoplasmic membrane that require energy (Krulwich, 1983). The organism can resolve this problem by substituting $\Delta\mu_{\text{Na}^+}$ generators for $\Delta\mu_{\text{H}^+}$ generators as in the case of *Vibrio alginolyticus* (Tokuda and Unemoto, 1982).

A possible explanation for the lack of evidence for sodium pumping in the terminal oxidase of the two strains of *Vitreoscilla* tested is the pH at which the organism was grown. *Vitreoscilla* strains used in this study were grown at a pH of 7.8. The NADH:quinone oxidoreductase from *Vibrio alginolyticus* has been shown to have an optimum pH of 8.5 for sodium pumping in whole cells and a minimum at pH of 6.00 to 6.5 (Tokuda and Unemoto, 1984). Webster's group, who reported that the terminal oxidase from *Vitreoscilla* pumps sodium, grew their strains at a pH of 8 having found that the optimum pH for sodium pumping was 8.5. At a pH of 7.8 Webster *et al* found about 86 % of the sodium pumping activity that they found at the optimum pH of 8.5 (Webster, Park, and Moon, 1995). It is therefore not likely that the pH at which the organism was grown was responsible for the lack of evidence of sodium pumping in the two strains of *Vitreoscilla* tested.

Vitreoscilla stercoraria has only been isolated from cow dung. The pH of cow dung was investigated (see Chapter 2 Page 39) and found to be 7.3; this compares with around 8.2 for seawater (Dring, 1982). Although the cow dung is slightly alkaline this may not be enough to provide a situation where utilisation of a primary sodium pump would be an advantage. The sodium content of cow dung was found to be 0.148 % of the wet weight; this is far lower than seawater (2.6 % by weight). Skulachev states that $\Delta\mu_{\text{Na}^+}$ can only support the performance of work if the Na^+ concentration outside the cell is greater than that inside (Skulachev, 1987). The combination of very slightly

alkaline pH and low sodium content in the tested dung make this a habitat where organisms are unlikely to use a primary sodium pump.

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Chapter 6

Summary of findings and prospects for future work

6.1 Selection of strain of *Vitreoscilla stercoraria* for use in the study

Although a strain of *Vitreoscilla* was supplied by Dr Dale Webster, for various reasons outlined in chapter 2 it was necessary to isolate a strain directly from the environment. Problems were encountered purifying the isolated strains because of contaminating bacteria that adhered to the mucus layer of the *Vitreoscilla*. A novel protocol was developed to purify the isolates using lysozyme. This was successful for two of the isolates and they were then screened for their affinity for sodium ions.

Strain LB13 was chosen for further work and the small scale (50 ml) growth was scaled up to 2 l flask. Shaking the flasks produced a low yield because the bacterial were unable to adhere to the surfaces; after some experimentation an aeration method was developed that involved pumping air straight into the broth. This method enabled the aeration of the flasks to take place with minimum agitation to the broth.

6.2 Growth of *Vitreoscilla stercoraria*

Although some work was carried out on optimising the growth condition for *Vitreoscilla* it is likely that an improvement could be made to the yield by employing a solid matrix such as glass beads to provide a greater surface for the bacteria to grow upon. The beads could be packed into glass tubes and the media dripped onto them; this would provide the aeration that the organism requires while providing an increased surface area for growth. The bacteria could then be removed from the beads by agitation and washing.

6.3 Disruption of cells

Once large scale growth was established, cells were harvested for isolation of respiratory membranes. Problems were encountered breaking the bacteria open with the Bead Beater and so lysozyme was added. Large quantities of DNA were released from the cells when they were broken. This made it impossible to pipette the solution and so DNAase was added to break down the DNA. This was not immediately successful and so EDTA was left out of the extraction buffer and MgSO_4 was added to the bead beater to provide a divalent cation for the DNAase. Development of this protocol took a number of months but eventually respiratory membranes were isolated that were of a quality suitable for use in the measurement of oxidase activity.

6.4 Quinol oxidase activity

The effect of sodium, lithium and potassium chloride on the quinol oxidase activity of the isolated membranes was tested using a number of different quinol substrates. At no point was the quinol oxidase activity of the membranes stimulated by the addition of sodium ions. *Vitreoscilla* respiratory membranes contain two quinol oxidases (Georgiou and Webster, 1987) and so the possibility existed that the activity of one was masking the increase in activity of the other. To test this, the experiments were repeated using membranes that had been treated with octylglucoside. The *bd* oxidase from *Escherichia coli* is inhibited by octylglucoside and, (Lorence, Miller et al., 1984) to determine that the *bd* from *Vitreoscilla* had been inhibited by octylglucoside quinol oxidase activities were carried out in the presence of cyanide which inhibits cytochrome *bo* but not *bd* (Pudek and Bragg, 1974). Again, during the quinol oxidase activity measurements no stimulation of quinol oxidase activity was observed.

Whilst the membrane quinol oxidase activity experiments were underway the development of a protocol for the purification of the terminal oxidase was also being developed, this was based on a method used to isolate cytochrome *bo* from *E. coli* RG145 (see Chapter 3, Figure 3.1 Page 73). An initial attempt to purify the enzyme by extracting with octylglucoside and then applying directly to an ion exchange column and therefore omitting the urea/cholate wash was unsuccessful because the enzyme failed to adhere to the column. The omitted urea cholate and ultracentrifugation step was therefore replaced and a second attempt proved successful. However the resulting yield was low. The next step was to run the enzyme through a hydrophobic interaction column. However, the low yield from the ion exchange column made this impractical, after the hydrophobic interaction column the yield was too low to be any use. The hydrophobic interaction column was therefore removed from the protocol and the enzyme was used as prepared after the ion exchange column.

Again quinol oxidase activity measurements were made and the effects of the addition of sodium, lithium and potassium chloride were tested. Once again no stimulation of activity was observed for any of the ions tested with any of the substrates tested.

6.5 Photosensitivity

During routine growth in the laboratory it was noted that cultures of *Vitreoscilla* LB13 inoculated in the evening grew well overnight whilst cultures inoculated in the morning failed to grow. This observation led to the discovery that *Vitreoscilla* is sensitive to

light. During an early experiment into the nature of this sensitivity a strain was isolated that showed resistance to the effect of UV. This strain (termed LB13a) was used as a comparison in later experimentation. Another strain of *Vitreoscilla* were tested and also found to be extremely sensitive to light. The light sensitive and insensitive strains of *Vitreoscilla* were compared to *E. coli* K12 for UV sensitivity. The insensitive strain showed a similar response to the *E. coli* whilst the sensitive strain viability was affected by the level of UV used.

It was probable that the strain of *Vitreoscilla* that was insensitive to the light had lost something that the other possessed. It is unlikely that a spontaneous mutation would produce a repair system or some other compound that would impart resistance. A possible candidate was an element of the electron transport chain where oxygen radicals may be produced and so the respiratory membranes of both the strains were compared.

Although differences were seen between the first set of membranes prepared, on subsequent preparations no difference was noted. The first preparation showed differences consistent with the presence of a flavoprotein in the membranes from the sensitive strain that was not present in the membranes from the resistant strain. *Vitreoscilla* contain components that are regulated by oxygen availability such as cytochrome *bd* in the respiratory membranes and a bacterial haemoglobin that is contained in the cytosol. It is possible that differences in aeration between the two cultures caused a difference in the ratio of components in the respiratory membranes of the two strains.

6.6 Sodium pumping

Vitreoscilla is the only bacterium that has been reported to possess a sodium pumping terminal oxidase that has been identified and partially characterised see Chapter 1, Page 22 (Bassey, Efiok et al., 1990; Bassey, Efiok et al., 1992; Bassey, Efiok, and Webster, 1990; Park, Moon et al., 1996).

The measurements of activity show that the terminal oxidase from *Vitreoscilla* is not sodium dependent and is therefore not likely to be a sodium pump, no evidence of sodium pumping was found in either LB13 or C1 (the strain reported to possess a sodium pumping terminal oxidase)

6.7 Interesting questions leading to further work

6.7.1 Gliding

Some interesting questions remain about the nature of gliding motility in general and in *Vitreoscilla* specifically. It was noted early in the study that *Vitreoscilla* strains repeatedly subcultured into broth lost the ability to glide when returned to solid media. Does this indicate that the ability to glide may be coded on a plasmid that can be lost without selection pressure or that the organism is simply switching off the gene when it is not needed ? The isolation method used may select for strains that were in trichomes, it has been reported that other species of gliding bacteria for example *Myxococcus xanthus* move quicker in chains than they do as single cells (Spormann and Kaiser, 1995) . Do the trichomes form from dividing bacteria or do the individual cells join to form them? Costerton *et al* reported that the individual cells may join to form chains but they may be simply following the mucous layer from the cell in front

(Costerton, Murray et al., 1961). If the cells do join up to form trichomes, do individuals from different strains join? If different strains do not join what is the mechanism that prevents this from happening? These questions could be answered with the use of fluorescence in situ hybridisation (FISH). This is a technique used to detect DNA or RNA sequences in cells, tissues or tumors (for a review of the method see Bouvier and del Giorgio, 2003). The technique allows the localisation of specific DNA sequences within the cell. Fluorescence labeled probes are designed against a specific target sequence in the DNA or RNA of the organism under examination, these target sequences can then be located using fluorescence microscopy (Nath and Johnson, 2000).

FISH could be used to investigate the gliding motility of *Vitreoscilla*. A strain of *Vitreoscilla* would be engineered that had a target sequence inserted. A deletion could also be distinguished from the wild type but in this case the wild type would be probed for. It would be preferable to produce two mutants from the same strain carrying a different target sequence so that each could be tagged with a probe that fluoresces at a different wavelength and could be visualised and distinguished. Alternatively fluorescent reporter genes such as green fluorescent protein (GFP) and yellow fluorescent protein (YFP) could be inserted. As a selection pressure is necessary to keep the reporter gene present in the genome of the organism it would be preferable to add the gene by recombination rather than using a plasmid vector; this would mean that the gene would be relatively permanent. The strains of *Vitreoscilla* could then be grown together on solid media, slides would be prepared from this mixed culture and trichomes could be examined to see if they were mixed. This would answer the question of whether the cells divide into trichomes or join to

form them. If all the trichomes were the same colour then the cells must have divided from a parent cell but if the trichomes contain cells tagged with different colours then they must have joined. The question of whether different strains join could then be answered using the same approach.

6.7.2 Light sensitivity

Further investigation is needed into the mechanism responsible for the extreme UV-A sensitivity of *Vitreoscilla*. A molecular approach could be used to identify the gene or genes responsible. Transposable genetic element mutation (transposon mutation) could be employed to identify the responsible gene. Transposons are small sequences of DNA that can move to any position in a cell's genome. The gene into which the transposon inserts is usually inactivated (Turner, McLennan et al., 1997). *Vitreoscilla* would be mutated using a transposon and the resulting mutants screened for light sensitivity. Any mutants no longer sensitive to light could be presumed to have a transposon inserted into the gene responsible for the light sensitivity. The genome of the resulting mutant would then be digested and separated using gel electrophoresis, the gel would be probed for the transposon and this would be amplified and inserted into a known plasmid. The resulting plasmid would be cloned into *E coli* and grown up in bulk. The plasmid could then be recovered and mapped and the gene sequenced. The sequence of the gene responsible for light sensitivity would now be known and a search of a data base of known gene sequences could be used to compare this sequence with those of other genes. This would give an indication of the protein coded by this sequence. Software is also available that would provide an indication of the structure of the protein (Evenden, 2003). For example a membrane protein might be identifiable because of the characteristic

hydropathy plots that they give i.e. hydrophobic regions (corresponding to transmembrane alpha helices) interspersed with relatively hydrophilic loops.

Once sequenced it would be possible to clone the gene into a host bacteria such as *Escherichia coli* and determine whether the resulting clone was rendered light sensitive by the gene. This would confirm that the gene was responsible for the light sensitivity in *Vitreoscilla* (Evenden, 2003).

Some transposons insert into more than one position in the genome, it would be preferable to use one that is self regulating and will only insert once into the genome, ensuring that the sequence amplified would be that of the desired gene. If more than one gene is responsible, then more than one type of mutant might be produced, examination of a number of mutants would determine whether one or more gene was responsible (Evenden, 2003).

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Appendix A

- A. 50 mM potassium phosphate pH, 7.5 with KOH
 - B. 50 mM potassium phosphate, 1mM EDTA, pH 7.5 with KOH
 - C. 50 mM potassium phosphate, 0.1 mM EDTA, 0.1% triton X100, pH 7.5 with KOH
 - D. 350 mM potassium phosphate, 0.1 mM EDTA, 0.1% triton X100, pH 7.5 with KOH
 - E. 25 mM tris.SO₄, 0.1 mM EDTA, 0.1% triton X100, pH 7.2
- 25 mM tris.SO₄, 250 mM K₂SO₄, 0.1 mM EDTA, 0.1 % triton X 100, pH 7.2

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In memory of
Jemma,
Pete and Sam

Extreme UV-A sensitivity of the filamentous gliding bacterium *Vitreoscilla stercoraria*

Laura F. Biggs, A. John Moody *

School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

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Abstract

Strains of the filamentous gliding bacterium *Vitreoscilla*, LB13 and C1, are shown to be highly sensitive to UV-A (320–400 nm), with an LD₅₀ of less than 20 kJ m⁻². *Vitreoscilla* LB13 can be protected from UV-A by including superoxide dismutase and catalase, separately or in combination, during the exposure, indicating an involvement of reactive oxygen species. LB13A, a photo-insensitive strain derived from LB13, is described.

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Keywords: *Vitreoscilla*; Gliding bacterium; Reactive oxygen species; UV-A; Photosensitivity

1. Introduction

Bacteria of the genus *Vitreoscilla* are Gram-negative chemoorganotrophic filamentous organisms that move by gliding [1]. *Vitreoscilla* have been classified in the past as colourless cyanobacteria [2] but they are not cyanobacteria, and are currently classified in the β purple bacteria [3]. Neither are they strictly colourless, since they have a respiratory chain that has both haemoprotein and flavoprotein components [4]. In addition, they express high levels of bacterial haemoglobin in their cytosol [5].

The work described here was prompted by a chance observation that a *Vitreoscilla stercoraria* isolate failed to grow when placed close to an external window, but not in direct sunlight, illumination conditions that had no noticeable effect on other organisms commonly being cultured in the same laboratory, e.g. *Escherichia coli*. Hence, it appeared that *Vitreoscilla* was particularly photosensitive, most probably to the UV component of sunlight. The aim of the work presented here was to investigate the photosensitivity of *V. stercoraria*. We show that the organism is sensitive to UV-A at low doses (LD₅₀ < 20 kJ

m⁻²), placing it among the most UV-sensitive bacteria yet described. The bacteriocidal effect of UV-A on *V. stercoraria* appears to involve UV-A-induced production of reactive oxygen species.

2. Materials and methods

Microbiological media came from Oxoid (Basingstoke, UK). Reagents came from Sigma (Poole, UK) unless otherwise stated.

Modified tryptone yeast (MTY) agar contained per litre: 12 g agar (technical agar number 3), 10 g yeast extract (L21), 10 g tryptone (L42) and 2.5 g sodium chloride. The pH was adjusted to 7.8 with sodium hydroxide. MTY broth contained the same components, except for the agar.

Vitreoscilla LB13 was isolated from cow dung on MTY agar. The dung was obtained from a site on Dartmoor (Devon, UK). LB13 was identified as a strain of *V. stercoraria* from its cell and colony morphology, including the presence of isolated motile trichomes and of mucus trails behind these trichomes [1].

To purify the organism, culture was dispersed in phosphate-buffered saline (Oxoid BR14a) containing 5 mg ml⁻¹ lysozyme (Sigma, L6876). After shaking for 10 s the suspension was incubated at 20°C for 15 min, and then shaken for 10 s. Loops of this suspension were streaked onto fresh MTY agar, which was incubated at

* Corresponding author. Fax: +44 (1752) 232970.

E-mail address: jmoody@plymouth.ac.uk (A.J. Moody).

Abbreviations: UV-A, ultraviolet radiation, wavelength range 320–400 nm; UV-B, ultraviolet radiation, wavelength range 286–320 nm

25°C for 24 h, after which colonies derived from single trichomes could be identified. Pre-treatment with lysozyme breaks up mucus that *Vitreoscilla* produce in their gliding motility, increasing the likelihood of obtaining isolated single colonies.

The light-insensitive strain LB13A arose from LB13 during a preliminary experiment in which the light sensitivity of LB13 was being investigated. Cultures of LB13 in MTY broth were being illuminated in universal bottles in a plant growth cabinet (MLR-350 HT; Sanyo Gallenkamp plc, Loughborough, UK; running with three banks of 3×40-W lamps; UV-A irradiance <0.22 W m⁻²). LB13A was isolated from a spread plate prepared from one of these broth cultures. Note that the spread plate was incubated under the same illumination, conditions under which LB13 does not grow. Strains LB13 and LB13A have the same cell and colony morphology; they appear to differ only in light sensitivity.

Vitreoscilla C1 (ATCC 13981) was a gift from Dr Dale Webster (Illinois Institute of Technology). *E. coli* K12 (ATCC 23716) was used as a control organism.

To prepare pre-cultures/cultures bacterial strains were streaked from liquid nitrogen storage onto MTY agar and incubated at 25°C for 48 h. Loops of culture were then transferred into 250-ml conical flasks containing 50 ml of MTY broth, and the flasks were incubated at 25°C for 48 h. Incubations were in the dark except for LB13A where the illumination conditions described above were used.

For irradiation of organisms with UV-A, all procedures were carried out on ice and samples were processed under low light conditions. Cells were harvested from pre-cultures (see above) by centrifugation (500×*g*_{av} for 10 min) and re-suspended in 0.9% NaCl. Ten millilitres of each suspension containing about 5×10⁷ cfu ml⁻¹ were placed in polystyrene tissue culture flasks (80-cm² Nunclon flasks, Nalge Nunc International, Denmark, experiment shown in Fig. 1; 25-cm² Falcon flasks, Becton Dickinson Europe, Meylan, France, experiment shown in Fig. 2) and exposed to a UV-A source (Model UVLS-28, UVP, Upland, CA, USA; peak output at 365 nm). The unweighted irradiance was determined using an SR9910 spectroradiometer (Macam Photometrics, Livingston, UK) by placing the detector at a distance from the source equivalent to the surface of the bacterial cultures. A flat piece of polystyrene cut from a culture flask was placed on top of the detector. The irradiance was essentially uniform over the surface that the flasks were placed on. Also, the flasks were rearranged randomly on this surface after every hour of exposure. Although the UV-A source used also produced a low level of UV-B this was filtered by the polystyrene culture flasks; UV-B irradiance (286–320 nm) expressed as a percentage of UV-A irradiance (320–400 nm) was 0.019% without and 0.003% with the polystyrene. After exposure to UV-A samples of cell suspension (50 µl) were removed and serially diluted with MTY broth in 10-fold steps for determi-

nation of the number of colony-forming units remaining. At each step diluted cell suspension (100 µl) was spread onto MTY agar plates (in triplicate). These were then incubated in the dark at 25°C for 24–48 h after which the colonies were counted.

For the medium-scale growth of *Vitreoscilla* LB13 and LB13A pre-cultures were prepared as described above. Two-litre flasks containing 700 ml of MTY broth were each inoculated with 10 ml of pre-culture, and the flasks incubated at 25°C in a model G25 orbital shaking incubator (New Brunswick Scientific, Edison, NJ, USA) at 50 rpm for 48 h. Air was pumped into the flasks through Whatman Hepa-Vents (pore size 0.3 µm; supplied by Merck Eurolab, Poole, UK) directly into the broth at a rate of 200 ml min⁻¹. Cells were harvested by centrifugation at 9000×*g*_{av} for 20 min at 4°C in an MSE 24B high-speed centrifuge (MSE Scientific Instruments, Crawley, UK), and washed by re-suspension to the original volume with 50 mM potassium phosphate buffer, pH 7.5, followed by re-centrifugation.

Cytosolic extracts and cell membranes from LB13 and LB13A were prepared as follows. Cells (10 g wet weight) were placed in the chamber (75 ml) of a bead beater (Bio-spec Products, Bartlesville, OK, USA) with 0.2 g lysozyme, 50 mg DNase I (Sigma DN25) and 67 µl of 10 mM MgSO₄. Glass beads (27 g of acid-washed, 425–600 µm diameter) were then added and the chamber was filled with 50 mM potassium phosphate, pH 7.5. The chamber was incubated at 25°C for 15 min, and then placed on ice for 10 min. The cells were then disrupted using 20×15-s bursts of the bead beater, with 45 s cooling between bursts. After disruption half a 'Complete' mixed protease inhibitor tablet (Roche Molecular Biochemicals, Lewes, UK) was added. The suspension was centrifuged at 3000×*g*_{av} at 4°C for 10 min to remove unbroken cells. To isolate the membranes the supernatant was centrifuged at 35 000×*g*_{av} at 4°C for 30 min. The pellet was re-suspended with 50 mM potassium phosphate, pH 7.5, containing 1 mM potassium EDTA. Both the membrane suspension and the supernatant, i.e. the cytosolic extract, were stored at -80°C. Protein contents were determined by the Lowry method (Sigma kit P5656).

3. Results and discussion

3.1. The effect of UV-A on survival of *Vitreoscilla* LB13

Vitreoscilla LB13 was exposed to UV-A under defined conditions as described in Section 2, and its survival was compared with that of the photo-insensitive strain LB13A (derived from LB13 during a preliminary experiment; see Section 2), together with *Vitreoscilla* strain C1 (ATCC 13981) and *E. coli* K12 (ATCC 23716). After irradiation samples were taken and the number of colony-forming units determined by plate count. Fig. 1 shows the percent-

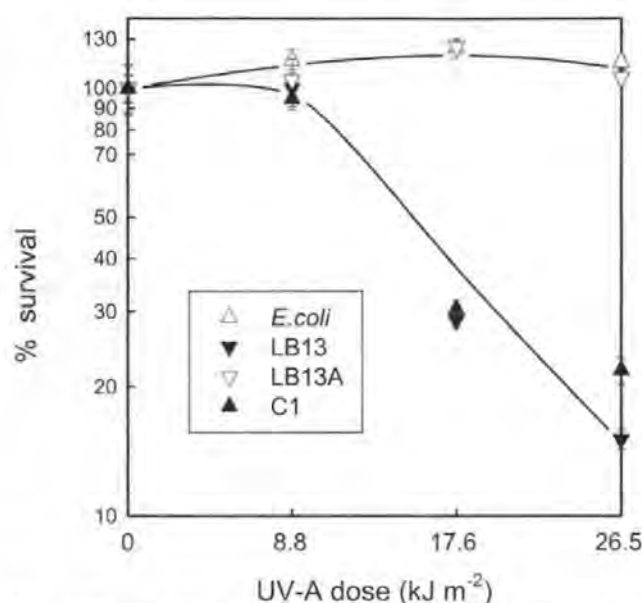


Fig. 1. Effects of UV-A on the survival of *E. coli* and three strains of *Vitreoscilla* (as indicated). Cell suspensions were kept on ice during the irradiation. Irradiance was 2.45 W m^{-2} . Percentage survival was determined based on the number of colony-forming units before and after irradiation. Mean data for three replicates \pm S.E.M. are shown.

age survival of each of the strains against UV-A dose. LB13 was UV-A-sensitive with an LD_{50} of about 15 kJ m^{-2} . C1 behaved in a similar way, but both *E. coli* K12 and LB13A were UV-A-insensitive. Both *Vitreoscilla* LB13 and C1 showed a threshold at about 9 kJ m^{-2} below which they were insensitive to UV-A under the conditions of this experiment.

3.2. Protection of *Vitreoscilla* LB13 against photodamage by UV-A

DNA absorbs and hence can be directly damaged by exposure to UV-C, and to a lesser extent UV-B [6], but this is not the case for UV-A [7]. Hence, the observation that *Vitreoscilla* LB13 is sensitive to UV-A suggests indirect damage. This could involve reactive oxygen species such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), generated through the reaction of an excited chromophore with molecular oxygen [7]. This was investigated by including the enzymes superoxide dismutase and catalase during the exposure of *Vitreoscilla* LB13 to UV-A. Fig. 2 shows that superoxide dismutase and catalase, separately or in combination, provided significant protection to the organism against damage by UV-A. Catalase absorbs UV-A strongly, so direct absorption could explain the protective effect. However, the mean transmission of UV-A by catalase at the concentration used was found to be 98.8% (pathlength = 4 mm), with a minimum of 97.8% and a maximum of 99.0%. Hence, direct absorption of UV-A by catalase could have had little protective effect.

The results with either superoxide dismutase or catalase separately are consistent with only partial protection by

these enzymes against UV-A. This suggests that both superoxide and hydrogen peroxide contribute to the UV-A-induced damage. However, superoxide seems to be more potent than hydrogen peroxide since superoxide dismutase provides protection despite one of the products of the dismutation of superoxide being hydrogen peroxide. Since externally added superoxide dismutase and catalase provided protection this perhaps implies that the site of their production as a result of UV-A exposure is on the 'surface' of the cells. However, both superoxide (in its protonated form, as the hydroperoxyl radical) and hydrogen peroxide can cross membranes [8], so equally the source could be internal.

3.3. Comparison of the chromophore content of *Vitreoscilla* LB13 and LB13A

Once the sensitivity of *Vitreoscilla* LB13 to UV-A was established the possibility arose that the chromophore responsible could be identified by comparison of the chromophore content of LB13 with that of LB13A, the photo-insensitive strain derived from it. The assumption was that exposure of LB13 to light had selected for a mutant that lacked the ability to produce this chromophore.

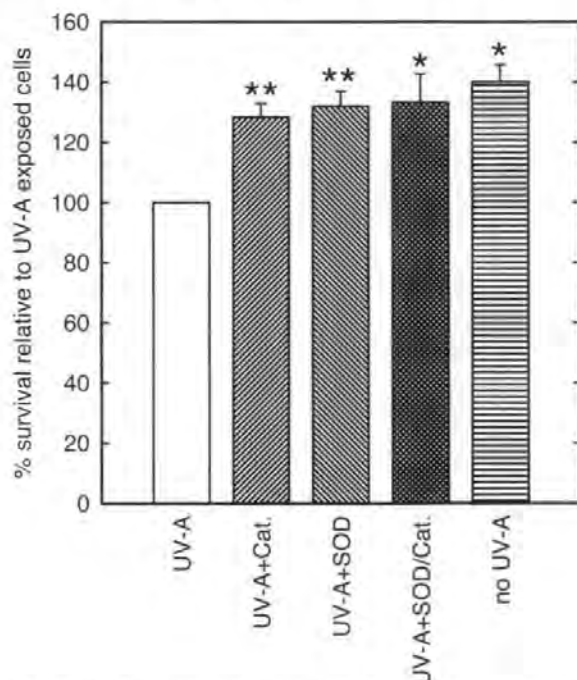


Fig. 2. Effects of superoxide dismutase (SOD) and catalase (Cat.) on the survival of *Vitreoscilla* LB13 after exposure to UV-A. Cell suspensions were kept on ice during the irradiation. Percentage survival was determined based on the number of colony-forming units before and after irradiation. Irradiance was 0.57 W m^{-2} ; dose was 6.2 kJ m^{-2} . Superoxide dismutase and catalase were present, as indicated, at 75 U ml^{-1} and 100 U ml^{-1} , respectively. Mean data for independent replicate experiments \pm S.E.M. are shown. One and two asterisks indicate significant differences at the 90% and 95% confidence levels, respectively, relative to UV-A exposure in the absence of superoxide dismutase or catalase (Student's *t*-test). In all cases $n=3$, except for no UV-A exposure, where $n=2$.

A characteristic of *Vitreoscilla* is high levels of bacterial haemoglobin in the cytosol [5]. Bacterial haemoglobin can be detected by using the change in the absorption spectrum induced by binding of carbon monoxide to the deoxy form of the protein. CO-binding spectra for cytosolic extracts from both LB13 and LB13A clearly show the presence of bacterial haemoglobin (data not shown); the specific contents were 57 and 40 nmol g⁻¹ of protein for extracts from LB13 and LB13A, respectively (using $\epsilon_{419-436\text{nm}} = 274 \text{ mM}^{-1} \text{ cm}^{-1}$ [5]). Hence the absence of this chromophore could not account for the photo-insensitivity of LB13A.

The dithionite-reduced minus air-oxidised difference spectra of both cytosolic extracts (data not shown) and membranes (data not shown) isolated from LB13 and LB13A were also examined. These showed no obvious qualitative difference in chromophore content. Membranes from both strains showed evidence of the presence of the terminal oxidases cytochromes *bo* and *bd* as indicated by the haem *b* α absorption band at 560 nm and by small absorption features between 600 and 700 nm [4]. Cytosolic extract and membranes from both strains also contained flavins.

3.4. The identity of the chromophore?

The potential role of flavins as chromophores involved in the lethal and sub-lethal effects of near-UV on bacteria has been discussed before [7]. Oxidised riboflavin, for example, has an absorption maximum (375 nm) that closely matches the UV-A used here (365 nm). The absorption of UV-A by a flavin could lead to its photoreduction [9], which could, in turn, cause direct damage by abstraction of electrons from components in its immediate environment. Alternatively this could cause indirect damage via the generation of superoxide (by donation of an electron to O₂ [10]), which appears to be the case here.

In a brief report of the photoinhibition of respiration in *V. stercoraria* [11], the rates of oxygen consumption by starved cells were measured after exposure of these to blue light ($\geq 400 \text{ nm}$). A dose-dependent decrease in rate was observed, with an *I*₅₀ of about 1000 kJ m⁻² (i.e. 10 min at $1.8 \times 10^6 \text{ erg cm}^{-2} \text{ s}^{-1}$). It was implied that the terminal oxidase cytochrome *bo* was the target for this photoinhibition, but no evidence was presented. Whether photoinhibition of respiration in *Vitreoscilla* can account for the killing effect of UV-A (Fig. 1) remains to be seen.

3.5. How sensitive is *V. stercoraria* to UV-A?

It is difficult to make absolute comparisons of the sensitivity of bacteria to UV radiation because of the different irradiation regimes used. However, there is certainly a range of sensitivity. Diverse responses to UV-B from a range of marine bacterial isolates have been found [12],

the most sensitive being *Vibrio natriegens* with an LD₅₀ value of about 100 kJ m⁻². Of three bacteria tested by Degiorgi et al. [13], *Pseudomonas aeruginosa* was the most sensitive to UV-B (LD₅₀ about 20 kJ m⁻²). Bacteriocidal doses of UV-A are likely to be higher. For example, Tyrell [14] reported data for *E. coli* consistent with approximate LD₅₀ values of 50 kJ m⁻², 250 kJ m⁻², and 1200 kJ m⁻² after exposure to UV with wavelengths of 313 nm, 334 nm and 365 nm, respectively. In conclusion, with an LD₅₀ of 15 kJ m⁻², it seems that *V. stercoraria* is particularly sensitive to UV-A compared to other bacteria.

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