The electron transfer chains of *Neisseria gonorrhoeae*

by

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Synopsis

Neisseria gonorrhoeae is an obligate human pathogen that can use both oxygen and nitrite as electron acceptors, but its electron transfer chain has only been partially characterised. The gonococcus encodes one azurin, Laz, and eight *c*-type cytochromes. The aim of this project was to determine the functions of these redox proteins.

Single mutants lacking cytochromes c_2, c_4 and c_5 reduced oxygen at 126%, 84% and 80% of the parental rate, respectively. It was not possible to construct a double mutant defective in both cytochromes c_4 and c_5, unless an ectopic copy of cytochrome c_5 was present, the expression of which was induced by IPTG. It was concluded that cytochromes c_4 and c_5 form a bifurcated electron transfer pathway between the cytochrome bc_1 complex and the cytochrome cbb_3 oxidase.

Candidates for electron donors to the nitrite reductase, AniA, were Laz and cytochromes c_2, c_4 and c_5. Mutants lacking various combinations of these redox proteins retained the ability to reduce nitrite, implicating the presence of further electron transfer pathways to AniA. Mutants defective in the third heme-binding domain of CcoP or the second domain of cytochrome c_5 reduced nitrite at 52% and 39% of the parental rate, respectively. The double mutant still reduced nitrite, but at only 12% of the parental rate. It was concluded that the third heme group of CcoP and the second heme group of cytochrome c_5 constitute the main electron transfer pathways to AniA, but a third pathway remains to be identified.

Previous research failed to demonstrate the presence of cytochrome c_2 on an SDS-PAGE gel stained for covalently bound heme. This was shown to be due to the low constitutive level of expression of this protein. Although a precise role for cytochrome c_2 could not be determined, the data presented suggest that the protein could function either as an electron donor to AniA, an electron donor to ScoI, or as a regulator of electron flux to the terminal reductases.

The combined data show that the plasticity of the gonococcal electron transfer chains allows this bacterium to respond rapidly to changes in terminal electron acceptor availability.

Dedication

To my family and Tom

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Abbreviations

Ascorbate-TMPD	Ascorbate N,N,N',N'-tetramethyl-ρ-phenylenediamine
ATP	Adenosine Triphosphate
cycA	gene encoding cytochrome c_4
cycB	gene encoding cytochrome c_5
cccA	gene encoding cytochrome c_2
Fe-S	non-heme iron-sulphur
GCA	Gonococcal Agar
GCB	Gonococcal Broth
IPTG	Isopropyl β-D-thiogalactopyranoside
OD	Optical Density
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1 Introduction

Neisseria species are members of the Betaproteobacteria class, Neisseriales order, *Neisseriaceae* family and the *Neisseria* genus. The majority of *Neisseria* species, such as *Neisseria cinerea, N. lactamica, N. mucosa* and *N. sicca,* are commensal human organisms that do not cause disease. Only two members of the *Neisseria* genus are pathogenic: *N. meningitidis* (referred to as the meningococcus), which inhabits the nasopharynx, and *N. gonorrhoeae* (referred to as the gonococcus), which inhabits the urogenitary tract. Whereas gonococci always cause infection in humans, meningococci can exist either as commensal organisms or cause the disease meningitis. The carriage rate of the meningococcus in the UK population is about 10%, and infection occurs in every 1 to 5 per 100,000 individuals in Europe (Virji, 2009). There are 5 major serogroups that cause epidemics: A, B, C, W-135 and Y. Vaccines exist against all serogroups except serogroup B due to its poorly immunogenic polysaccharide capsule (Sadarangani & Pollard, 2010).

Neisseria gonorrhoeae is a Gram negative, oxidase positive diplococcus and is the etiological agent of the disease gonorrhoea. It is an obligate human pathogen that principally inhabits the urogenital tract of men and women, often being asymptomatic in the latter. The bacterium invades epithelial cells of the genitourinary tract and can survive intracellularly (Edwards & Apicella, 2004). If left untreated in women, gonorrhoea can result in pelvic inflammatory disease and infertility (Nassif *et al.*, 1999). Gonococcal infections are usually localised, but can occasionally disseminate into the blood stream to cause endocarditis and meningitis (Edwards & Apicella, 2004). Gonorrhoea is the second most common sexually transmitted infection in the UK, and the incidence of gonorrhoea in the United Kingdom in

2005 was 165 per 100,000 individuals (Moran, 2007). There are currently no vaccines available against *N. gonorrhoeae* due to the high level of antigenic and phase variation in potential vaccine targets.

Despite the clinical importance of gonorrhoea in human health, not much is known about how the bacterium exploits energy sources to allow it to thrive within the human host. Although there have been some preliminary studies of both gonococcal and meningococcal electron transfer chains, the literature is confusing and incomplete. The objective of this project was to identify the functions of electron transfer chain components encoded by the gonococcus and, more specifically, to determine the pathways of electron transfer to the terminal electron acceptors oxygen and nitrite. In this chapter, the pathogenicity factors and mechanisms used by the gonococcus to evade the host immune system and protect itself from oxidative and nitrosative stress will be described. Oxidative phosphorylation and denitrification pathways will then be discussed and current knowledge of components of these electron transfer pathways will be summarised. Finally, the aims of this project will be stated.

1.2 Pathogenicity and evasion of host defence mechanisms

In order to interact with and adhere to epithelial cells, gonococci use pili, opacity-associated adhesin proteins (Opa) and porins. The gonococcus encodes two porins, Por1A and Por1B, which interfere with cell signalling and, together with pilin and Opa proteins, allow the bacterium to invade epithelial cells. This results in a local inflammatory response including the recruitment of polymorphonuclear leukocytes (PMN) and macrophages, both of which

phagocytose gonococci (Virji, 2009). Despite the harsh conditions, gonococci are able to survive within these phagocytic cells (Criss & Seifert, 2008; Criss *et al.*, 2009).

The gonococcus has evolved many mechanisms to evade attack by the host immune system. Gonococci are able to produce excess membrane, which forms into vesicles or "blebs". It is believed that membrane "blebbing" acts as a decoy, causing complement and antibodies to be diverted away from the bacterium (Pettit & Judd, 1992). Some proteins such as pilin and opacity proteins are subject to both antigenic and phase variation (Stern *et al.*, 1986; Hill & Davies, 2009). Both of these methods prevent the host immune system from recognising the bacterium. Molecular mimicry of host carbohydrates is another system by which bacteria can conceal themselves from the adaptive immune response. It was observed that gonococci can use α-2,3 sialyltransferase to sialylate lipopolysaccharides (LPS) on their outer membrane using CMP-NANA, present in human serum, as a substrate (Nairn *et al.*, 1988; Parsons *et al.*, 1988; Parsons *et al.*, 1989; Parsons *et al.*, 1992; Smith *et al.*, 1992). Further research revealed that gonococcal lipo-oligosaccharides (LOS) can also mimic human glycosphingolipids (Mandrell *et al.*, 1988; Mandrell, 1992; Mandrell & Apicella, 1993). Sialylated LOS and gonococcal porin (specifically Por1A) are able to bind human factor H protein, a regulator of the complement system (Ram *et al.*, 1998a; Ram *et al.*, 1998b). The complement system constitutes part of the innate immune system and causes bacterial cell death through the use of the membrane attack complex (MAC), which forms channels in the bacterial membrane and results in bacterial lysis (Müller-Eberhard, 1986). The C3b protein is deposited onto bacteria to aid in the phagocytosis of bacteria by opsonisation, and is also required for the formation of the MAC. Factor H cleaves C3b into iC3b and thus inactivates

C3b and the complement system. It is believed that the gonococcus coats itself in factor H to reduce the activation of complement, thereby protecting the gonococcus against part of the innate immune system (Welsch & Ram, 2008).

N. gonorrhoeae has evolved and acquired many mechanisms to protect itself against a wide range of antibiotics and chemotherapeutic agents. Sulphonamides were used against gonococci in 1935, but by 1944 resistance to this group of agents was noted (Anon, 1944). Penicillins were used as an alternative from 1943, but by 1976 many strains of gonococci had acquired mechanisms to protect themselves against these antibiotics (Percival *et al.*, 1976). Many gonococcal strains are now resistant to tetracyclines, aminoglycosides (such as gentamycin, kanamycin and streptomycin) and macrolides such as erythromycin (Lewis, 2010), all of which target the bacterial ribosomes to prevent protein synthesis. Gonococci are able to protect themselves from these antibiotics by altering their ribosomes by point mutation of the ribosomal coding regions as well as by modification of the ribosomes by methylation. The bacteria also encode a multiple transferable resistance efflux pump (MtrCDE) that is able to expel penicillin, tetracycline and macrolides (Shafer *et al.*, 2001). Many strains of gonorrhoea are resistant to multiple antibiotics and chemotherapeutic agents, and currently the only agents available against gonorrhoea are quinolones (which target DNA gyrase) and cephalosporins (which, like penicillins, inhibit the synthesis of the peptidoglycan layer) (Lewis, 2010).

It has been observed that bacteria are more resistant to antibiotics when bound in biofilms compared to when they exist in planktonic form (Ceri *et al.*, 1999; Dunne, 2002). Biofilms are

communities of micro-organisms that are embedded within a glycocalyx, which surrounds the bacteria and provides both protection and secure attachment to the surface. Biofilms form on surfaces where the habitat is favourable in terms of nutrient availability and suitability of the environment for bacterial growth and survival. Although biofilm formation by gonococci has not been extensively studied, it has been shown *in vitro* that gonococci can form biofilms on cervical epithelial cells (Steichen *et al.*, 2008). Unlike biofilms produced by other bacteria, which consist of exopolysaccharides and water, the glycocalyx produced by the gonococcus consists of membrane "blebs", which are believed to contain DNA (Dorward *et al.*, 1989; Dunne, 2002; Greiner *et al.*, 2005). It is thought that DNA contained within the "blebs" is released and constitutes a major component of the biofilm (Steichen *et al.*, 2011).

1.3 Oxidative and nitrosative stress protection mechanisms

Neisseria gonorrhoeae colonises the urogenital tract, where it is then able to infect the epithelial cells and survive within polymorphonuclear leukocytes (Edwards & Apicella, 2004; Simons *et al.*, 2005; Virji, 2009). During both colonisation and infection stages the gonococcus is exposed to reactive oxygen and nitrogen species, which can cause damage to DNA, proteins and lipids (Storz & Imlay, 1999). Sources of oxidative and nitrosative stress include (i) the host immune response; (ii) products of metabolism of commensal organisms inhabiting the same niche as the gonococcus; and (iii) products of gonococcal metabolism. Although the gonococcus is able to conceal itself from the adaptive immune system, the host is able to employ its innate immune system against the bacterium. The presence of gonococci often results in a local inflammatory response, where polymorphonuclear leukocytes (PMN) are recruited. These phagocytes ingest and attack the bacteria by

generating a respiratory burst using NADPH oxidase (Lee *et al.*, 2003; Simons *et al.*, 2005). The reactive oxygen species produced by this process include superoxide, hydrogen peroxide and hypochlorous acid. The metabolism of commensal organisms and gonococci themselves is another source of oxidative and nitrosative stress. *Lactobacilli*, which are part of the normal flora in the female genitourinary tract, produce hydrogen peroxide and nitric oxide among other reactive oxygen and nitrogen species. By-products of gonococcal respiration such as superoxide and hydrogen peroxide can be produced by some components of the respiratory chain, namely the NADH dehydrogenase, succinate dehydrogenase and cytochrome bc_1 complex (Seib *et al.*, 2006). The iron-sulphur centres of proteins can be damaged by both reactive oxygen and nitrogen species, but this damage can be repaired by DnrN, a gonococcal homologue of the *E. coli* YtfE protein and the *Staphylococcus aureus* ScdA protein (Rodionov *et al.*, 2005; Overton *et al.*, 2008).

The gonococcus has many defence mechanisms to protect itself against reactive oxygen species. It has a highly active catalase protein (encoded by the *kat* gene) that reduces hydrogen peroxide to water and is localised to the cytoplasm (Johnson *et al.*, 1993). The gonococcus also encodes a cytochrome *c* peroxidase (CCP), an outer membrane lipoprotein that reduces organic hydroperoxides and hydrogen peroxide to water (Turner *et al.*, 2003). It is possible that CCP could act as a terminal electron acceptor for an energy-conserving electron transfer chain (Seib *et al.*, 2006). In many bacteria, superoxide dismutases (Sod) are used to reduce superoxide to hydrogen peroxide, which in turn is reduced by catalases and peroxidases to water (Seib *et al.*, 2006). The gonococcus encodes a cytoplasmic iron-dependent superoxide dismutase, SodB, but this only has a low activity and is probably not a

major defence mechanism against superoxide in this organism (Archibald & Duong, 1986). The manganese uptake system (encoded by MntABC) functions independently from all of the aforementioned oxidative stress defences (Tseng *et al.*, 2001; Seib *et al.*, 2004). Manganese and manganese compounds are able to non-enzymically detoxify reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals (reviewed in Seib *et al*., 2004). The gonococcus therefore accumulates manganese using a specialised manganese uptake system in order to quench these reactive oxygen species.

The main reactive nitrogen species encountered by the gonococcus is nitric oxide (NO), which has a half life *in vivo* of less than one second (Pacher *et al.*, 2007). Nitric oxide is able to form further toxic compounds by reacting with molecular oxygen and superoxide, the latter reaction producing peroxynitrite which can damage metal cofactors of enzymes (Pacher *et al.*, 2007). Human cells can produce nitric oxide using nitric oxide synthases (NOS). These NOS use reducing power from NADPH to convert L-arginine and molecular oxygen into citrulline and nitric oxide, allowing the formation of further reactive nitrogen species such as peroxynitrite. Three types of NOS are found in the human host: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Alderton *et al.*, 2001). The eNOS and nNOS produce a basal, constitutive level of NO that is anti-inflammatory, whereas the expression of the cytosolic iNOS is induced by cytokines, which are produced as part of the host immune response (Alderton *et al.*, 2001). Polymorphonuclear leukocytes (PMNs) use the iNOS to produce proinflammatory levels of NO to attack invading pathogens (Tsukahara *et al.*, 1998). Nitric oxide is also produced during the reduction of nitrite by the copper-containing nitrite reductase, AniA (Mellies *et al.*, 1997; Householder *et al.*, 1999). The nitric

oxide reductase, NorB, is localised to the cytoplasmic membrane and reduces this nitric oxide to nitrous oxide (Householder *et al.*, 2000). Cytochrome *c*' (CycP) binds nitric oxide *in vitro*, and so it is believed to protect the gonococcus against this type of reactive nitrogen species (Turner *et al.*, 2005). As the expression of *cycP* is not regulated by the presence of NO, it is believed that CycP acts as a constitutive nitric oxide protection system, as distinct from the inducible NorB system (Stevanin *et al.*, 2005; Turner *et al.*, 2005; Heurlier *et al.*, 2008). Nitric oxide is a signalling molecule in the human host and, at concentrations greater than 1 μM, instigates an inflammatory response. The gonococcus is able to establish and maintain an NO steady state below this concentration, which might contribute towards the bacterium's ability to evade the innate immune system of the host (Cardinale & Clark, 2005).

1.4 Oxidative phosphorylation

Neisseria gonorrhoeae can use glucose, pyruvate, lactate or succinate as electron donors (Morse & Bartenstein, 1974; Winter & Morse, 1975). Under anaerobic conditions, glucose is fermented to produce lactate and acetate (Morse *et al.*, 1974). Under aerobic conditions (i.e. when oxygen is being used as the terminal electron acceptor), all of the components required for oxidative phosphorylation are found on the inner membrane. The pathway of electron transfer begins with the reduction of ubiquinone, a membrane-soluble lipid. The gonococcus has two NADH dehydrogenases that couple the oxidation of NADH to NAD^+ with the reduction of ubiquinone to ubiquinol (Figure 1.1). Nuo is a proton-translocating NADH dehydrogenase, and Nqr is a sodium-translocating NADH-dehydrogenase. Electrons gained by the NADH dehydrogenase from NADH pass through the multiple redox carriers of the dehydrogenase, namely FMN (flavin mononucleotide) and iron-sulphur centres. Ubiquinone

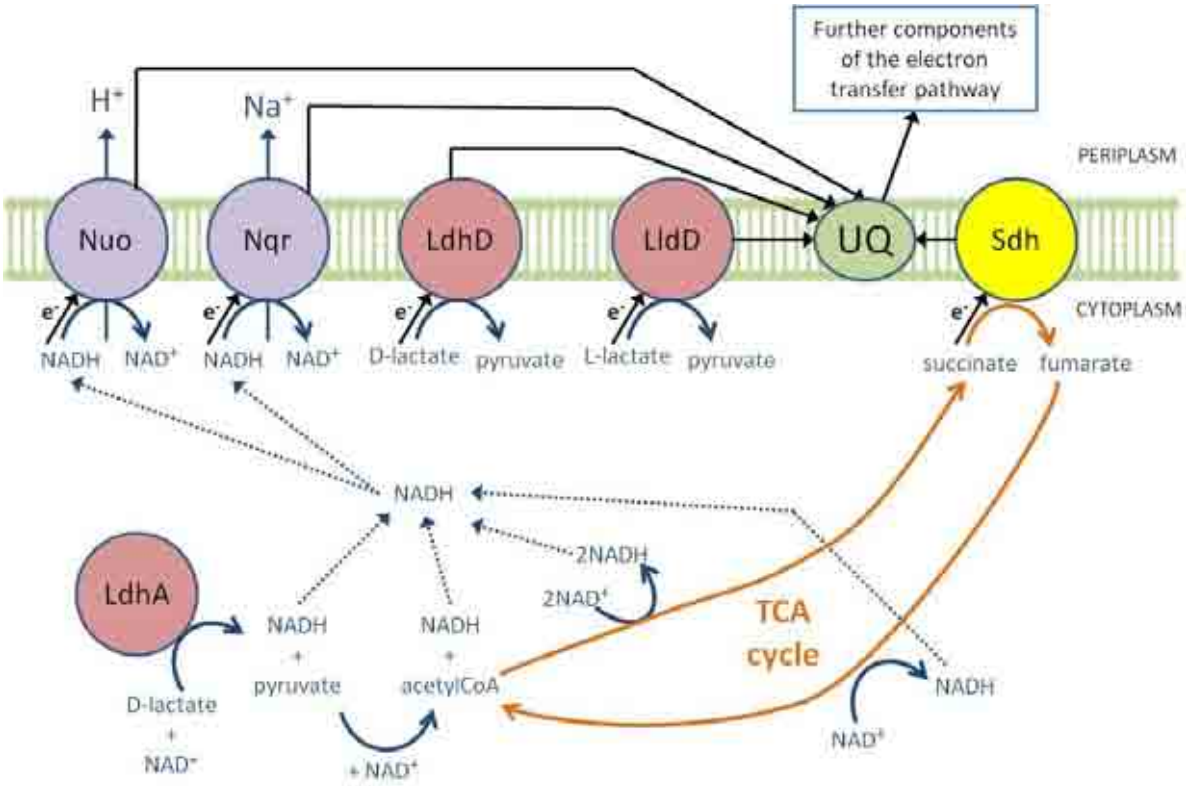

Figure 1.1 – Electron donors to ubiquinone in *Neisseria gonorrhoeae*

Black arrows represent electron transfer. Nuo and Nqr represent NADH dehydrogenase complexes. Sdh represents succinate dehydrogenase. LdhD, LldD and LdhA represent lactate dehydrogenases.

randomly diffuses laterally within the plane of the membrane, where it is eventually reduced by NADH dehydrogenase, resulting in the formation of ubiquinol. For every two electrons that are passed to ubiquinone, four protons are translocated by Nuo across the inner membrane into the periplasm. The oxidation of NADH by NADH dehydrogenase produces NAD^+, which is regenerated into NADH by components of the tricarboxylic acid (TCA) cycle.

The two NADH dehydrogenases are not the only electron donors to ubiquinone. Succinate dehydrogenase (Complex II) and two of the three lactate dehydrogenases encoded by the gonococcus can also reduce ubiquinone (Winter & Morse, 1975; Fischer *et al.*, 1994). As well as being an enzyme in the TCA cycle, succinate dehydrogenase (Sdh) is also a component of the electron transfer chain. It couples the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol (Figure 1.1). One of the lactate dehydrogenases, encoded by the *ldhA* gene, is a soluble, cytoplasmic protein, and its function is NAD^+-dependent. It catalyses the oxidation of lactate into pyruvate, coupled to the reduction of NAD^+ into NADH. The NADH produced by this reaction can then be used as a substrate by the NADH dehydrogenases. The other two lactate dehydrogenases, encoded by the *ldhD* and *lldD* genes, are membrane-bound, NAD^+-independent enzymes that couple the oxidation of lactate to electron transfer (Figure 1.1). The LdhD protein is only able to oxidise D-lactate, whereas the LldD protein is able to oxidise only L-isomers of lactate, phenyllactate and 4-hydroxyphenyllactate (Fischer *et al.*, 1994). In the absence of glucose, lactate is a substrate for the gluconeogensis pathway, allowing the production of glycerol by the bacterium. However, in the presence of glucose, gluconeogenesis is inhibited, allowing any lactate present in the medium to be used directly as an electron donor. Therefore, in media

containing glucose, lactate stimulates the metabolism of the gonococcus (Smith *et al.*, 2001).

Ubiquinone is bound by the aforementioned dehydrogenases, which reduce it to produce ubiquinol. Although the identity of electron donors to ubiquinone has been studied, downstream electron transfer chain components and their organisation have not been determined for *N. gonorrhoeae*. Sequencing of *N. gonorrhoeae* strains FA1090 and F62 has revealed that the bacterium encodes a cytochrome bc_1 complex and a single cytochrome *c* oxidase of cbb_3-type (GenBank accession numbers AE004969 and ADAA00000000, respectively). The cytochrome bc_1 complex consists of two *b*-type hemes, a Rieske iron-sulphur centre and cytochrome c_1 (Figure 1.2). Ubiquinol reduces the Q_o site of cytochrome *b*, which provides the low potential *b*-type heme with two electrons. One electron passes to the high potential *b*-type heme, where it partially reduces ubiquinone to semi-ubiquinone. The purpose of this chain is to eventually regenerate ubiquinol from ubiquinone. The other electron is transferred to the Rieske iron-sulphur protein, which rotates and reduces cytochrome c_1. As ubiquinone requires two electrons to be completely reduced to ubiquinol, two turnovers are required at the Q_o site. During each turnover, two protons from each ubiquinol molecule are translocated to the periplasm. This means that two turnovers of ubiquinol at the Q_o site results in the translocation of four protons to the periplasm, thus contributing to proton motive force (Trumpower, 1990; Crofts, 2004). During the second turnover, one electron again passes from the low potential *b*-type heme to the high potential *b*-type heme, where the bound semi-ubiquinone molecule is fully reduced to ubiquinol. In this way, for every two turnovers at the Q_o site, one molecule of ubiquinol is regenerated from ubiquinone, which requires two protons to be taken up from the

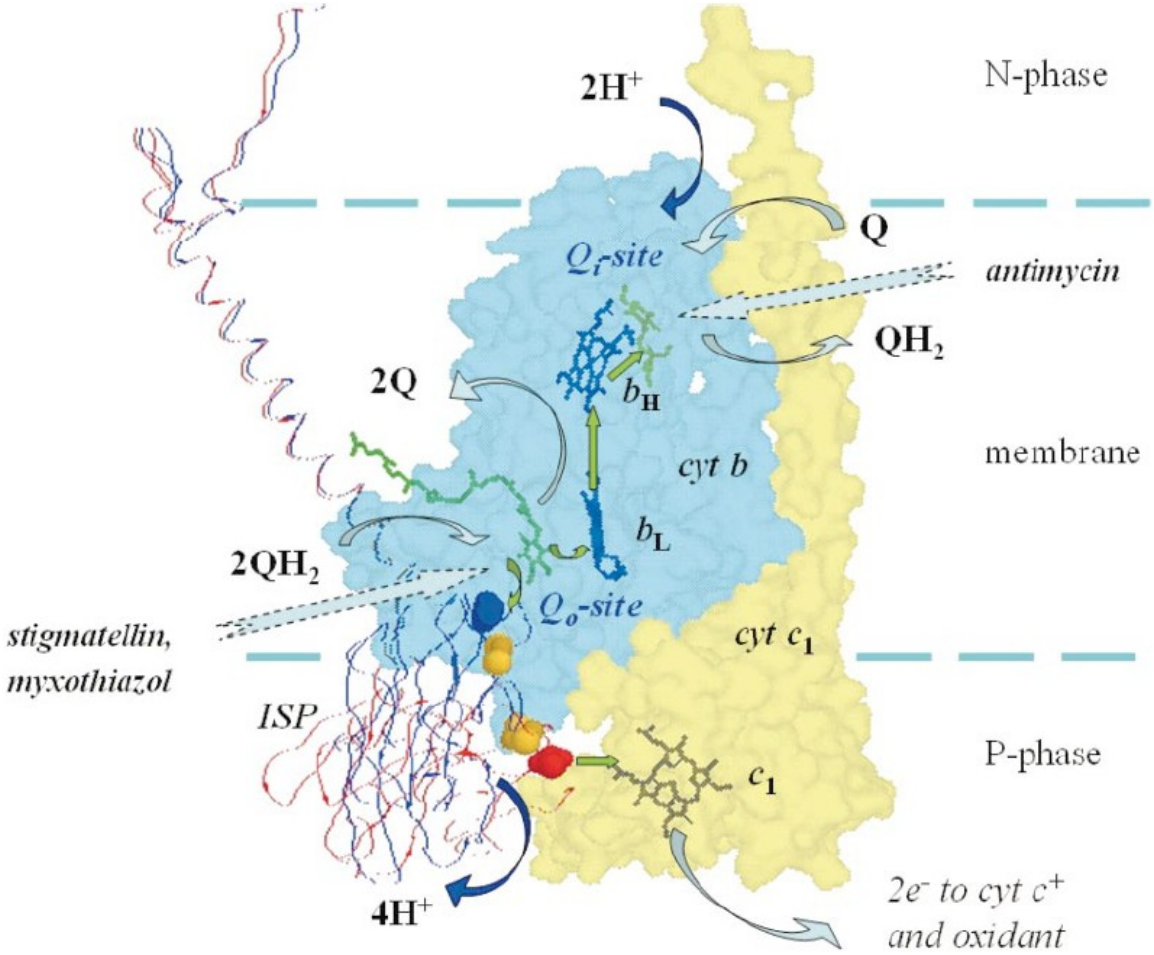

Figure 1.2 – Structure of the cytochrome *bc*$_1$ complex from chicken mitochondria

Cytochrome *b* is shown in blue, cytochrome c_1 in yellow, and the Rieske iron-sulphur protein is shown as a ribbon diagram. Green arrows indicate electron transfer, and curved blue arrows indicate proton movements. Dotted blue arrows indicate binding sites for inhibitors. P-phase indicates the periplasm, and N-phase indicates the cytoplasm. Adapted from Crofts, 2004.

cytoplasm. The other electron from the second turnover passes again to cytochrome c_1, therefore for every two turnovers at the Q_o site, two electrons are transferred to cytochrome c_1 (Crofts, 2004).

In organisms that encode a cytochrome bc_1 complex and a cbb_3-type oxidase, electrons are often transferred via small electron carriers (Figure 1.3). For example, in *Rhodobacter capsulatus*, which encodes only a cbb_3-type terminal oxidase, electrons are transferred via the mobile cytochrome c_2 and the membrane-bound cytochrome c_y (Daldal *et al.*, 1986; Jenney & Daldal, 1993). In *Azotobacter vinelandii,* cytochromes c_4 and c_5 fulfil this same role (Bertsova & Bogachev, 2002). Once reduced by the cytochrome bc_1 complex, the small cytochrome *c* is released from the cytochrome bc_1 complex and binds to the cytochrome cbb_3 oxidase. Molecular oxygen also binds to the cbb_3 complex, where it is reduced to water using four electrons donated from four reduced cytochrome *c* molecules. During this process, protons are pumped across the inner membrane into the periplasm by the cbb_3-type oxidase (Arslan *et al.*, 2000). The number of protons pumped per electron accepted varies between the different types of terminal oxidases. For example, aa_3-type oxidases have an H^+/e^- ratio of 1, whereas cbb_3-type oxidases have an H^+/e^- ratio of between 0.2 and 0.5. This suggests that cbb_3-type oxidases transduce less energy than aa_3-type oxidases (Pitcher & Watmough, 2004). An electrochemical gradient builds up between the periplasm and the cytoplasm due to the higher proton concentration in the periplasm. The gradient is dissipated by the passage of protons back into the cytoplasm via ATP synthase. When protons pass through ATP synthase, energy is provided for the ATP synthase to convert ADP and phosphate into ATP, which the cell can use as a source of energy (Anraku, 1988).

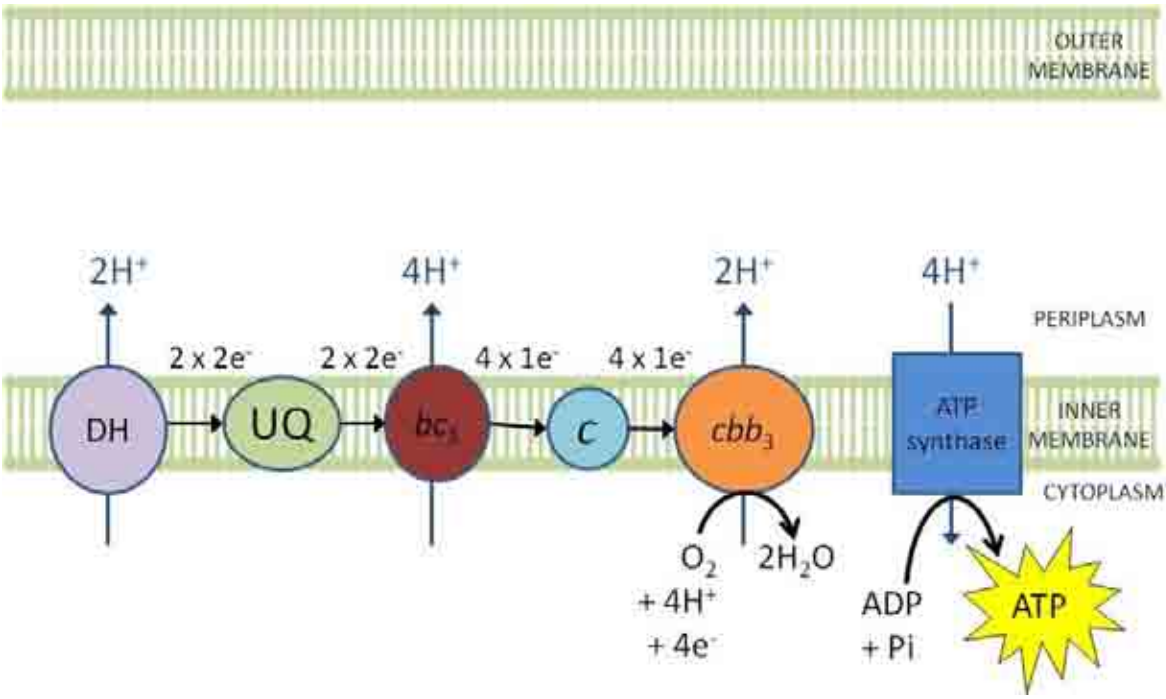

Figure 1.3 – Schematic of a typical bacterial electron transfer chain

Black arrows represent electron transfer. DH represents dehydrogenases (NADH dehydrogenases, succinate dehydrogenase, lactate dehydrogenases). UQ represents ubiquinone. bc_1 represents cytochrome bc_1 complex. *c* represents soluble cytochrome *c*. cbb_3 represents cytochrome cbb_3 complex.

Although the number of ATP molecules generated per proton translocated varies slightly between organisms, the H^+/ATP ratio is believed to be approximately 4 (Turina *et al.*, 2003).

When the proton concentration in the periplasm becomes far greater than in the cytoplasm, the dissipation of this gradient becomes limited by the rate of proton translocation through the ATP synthase. This results in the ubiquinol pool becoming over-reduced, which can decrease the efficiency of electron transfer from the cytochrome bc_1 complex to downstream redox proteins (Richardson, 2000). To prevent this, many bacteria can synthesise periplasmic rather than cytoplasmic terminal reductases that do not generate a proton motive force. For example, *Rhodobacter capsulatus* and *R. sphaeroides* encode periplasmic nitrate reductases that reduce nitrate without coupling this reaction to proton translocation into the periplasm (Ferguson *et al.*, 1987; Jones *et al.*, 1990). This allows the reduction of terminal electron acceptors to be uncoupled from ATP generation, and therefore prevents the ubiquinol pool from becoming over-reduced.

1.5 Cytochrome *cbb*$_3$ oxidases as members of the heme-copper oxidase family

Most oxidases of electron transfer chains are members of the heme-copper oxidase family, with the exception of cytochromes *bd*, which do not contain copper atoms (Anraku & Gennis, 1987; Anraku, 1988; Van Der Oost *et al.*, 1994). Members of the heme-copper oxidase (HCO) family all share a set of common features, namely their core catalytic subunit I and the presence of at least one additional subunit (Pereira *et al.*, 2001). The reduction of dioxygen to water and the resulting proton translocation by subunit I occurs in all HCOs.

Members of this family are thus functionally homologous. Heme-copper oxidases can have diverse electron donors and heme types. However, it is the subunit composition of a heme-copper oxidase that allows its classification into one of 3 major groups: type A, mitochondrial-like or aa_3-type oxidases; type B, ba_3-type oxidases; or type C, cbb_3-type oxidases (Pereira *et al.*, 2001). The cbb_3-type oxidases are the most divergent members of the heme-copper oxidase (HCO) family (Pitcher & Watmough, 2004).

The first cbb_3-type oxidase was discovered in *Bradyrhizobium japonicum* (Thöny-Meyer *et al.*, 1989). This bacterium forms a symbiotic relationship within the root nodules of the soybean, where it is able to fix nitrogen gas (N_2) using the nitrogenase enzyme. This enzyme is destroyed by oxygen, so it can function only when oxygen tensions are low. To achieve this, the bacterium expresses a cbb_3-type oxidase (encoded by the *fixNOPQ* gene cluster) that has a very low K_m for oxygen. This allows the bacterium to efficiently scavenge oxygen from the environment to provide conditions under which the nitrogenase enzyme can function, whilst coupling the reduction of oxygen to the generation of proton motive force (Preisig *et al.*, 1993; Preisig *et al.*, 1996). Cytochrome cbb_3 oxidases are often found in bacteria that inhabit microaerobic environments. As a result, many human pathogens such as *Helicobacter pylori* and members of the *Neisseria* genus encode a cbb_3-type oxidase (Tomb *et al.*, 1997; Parkhill *et al.*, 2000).

Cytochrome cbb_3 oxidases are composed of three subunits. The *ccoN* gene encodes the catalytic subunit I of the oxidase, where oxygen is reduced to water. Subunits II and III are *c*-type cytochromes encoded by the genes *ccoO* and *ccoP*, respectively (Saraste & Castresana,

1994). Unlike the CcoN subunit, which is embedded in the membrane, CcoO and CcoP are bound to the membrane but protrude into the periplasm. The structure of the *Pseudomonas stutzeri* cbb_3 oxidase has recently been solved, which has allowed the route of electron flow through the complex to be predicted (Figure 1.4) (Buschmann *et al.*, 2010). Electrons from a soluble *c*-type cytochrome enter the complex at the second heme group of CcoP and are transferred to the first heme group of CcoP. The electrons then flow to the single heme group of the CcoO subunit, after which they enter the CcoN subunit at the heme *b* site. From here, electrons pass to the heme b_3 site, which is oxidised by the Cu_B site. Electrons are thus available to the catalytic centre of the CcoN subunit, where oxygen can be reduced to water. A fourth subunit, CcoQ, is also associated with the cytochrome cbb_3 complex, although it does not form part of the core enzyme. While its role is unclear, in *Rhodobacter sphaeroides* it was thought that CcoQ was involved in a signal transduction pathway to enable the bacterium to regulate the expression of genes required for photosynthesis under low oxygen conditions (Oh & Kaplan, 2000). However, it has since been shown that CcoQ protects and stabilises the core cbb_3 enzyme (Oh & Kaplan, 2002), and that CcoP and CcoQ interact (Peters *et al.*, 2008). Therefore, it is now believed that CcoQ has only an indirect effect on the regulation of photosynthesis genes in *Rhodobacter sphaeroides.*

1.6 Denitrification processes in the *Neisseria* genus

Denitrification is the sequential reduction of nitrate to molecular nitrogen. In many organisms, the denitrification pathway contains a nitrate reductase, a nitrite reductase, a nitric oxide reductase and a nitrous oxide reductase. *Neisseria mucosa*, a commensal

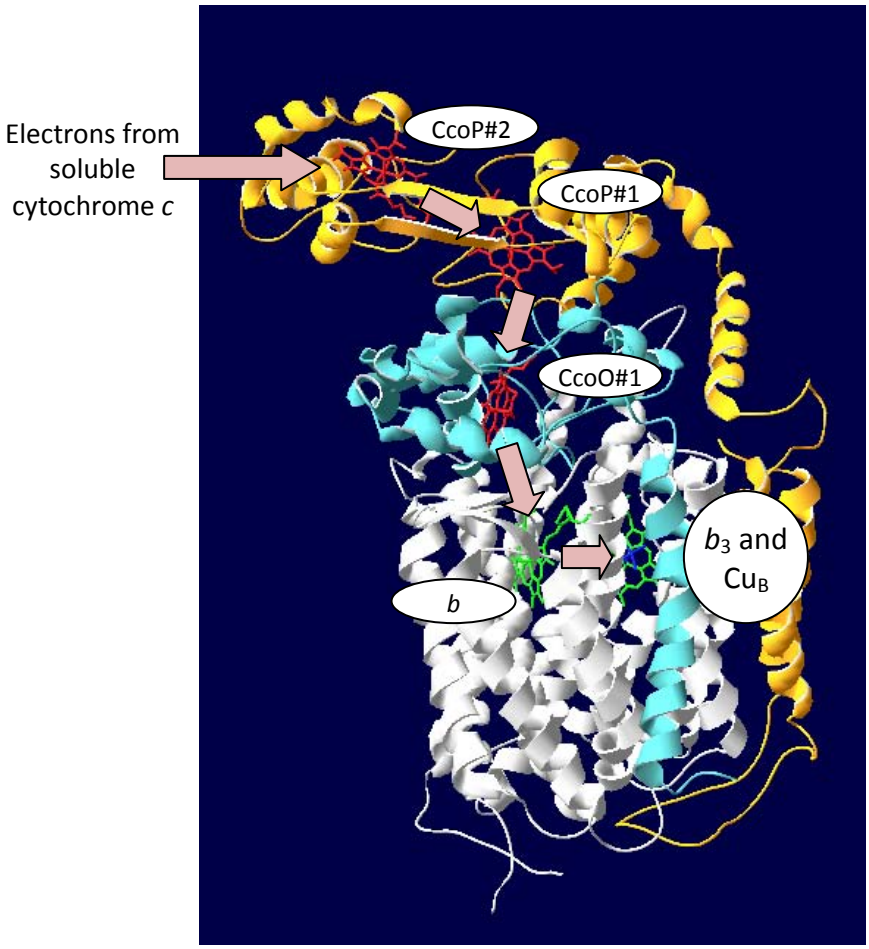

Figure 1.4 – Structure of the cytochrome *cbb*$_3$-type oxidase from *Pseudomonas stutzeri*

The CcoN subunit is shown in white, the CcoO subunit is shown in light blue, and the CcoP subunit is shown in orange. *c*-type heme groups are numbered and shown in red, *b*-type heme groups are numbered and shown in green, and Cu_B is shown in blue. Electron flow is indicated by pink arrows. Modified from PDB entry 3MK7 (Buschmann *et al.*, 2010) using the Swiss-PdbViewer program (Guex & Peitsch, 1997).

species, is the only member of the *Neisseria* genus found so far that is able to reduce nitrate to nitrite via a NarGHI complex (Barth *et al.*, 2009). The other members of the *Neisseria* genus do not have a nitrate reductase. Their denitrification pathways therefore begin with the reduction of nitrite to nitric oxide by nitrite reductase. The most abundant type of nitrite reductase in bacteria is cytochrome cd_1, encoded by *nirS*. These nitrite reductases function as homodimers and contain hemes *c* and d_1 as their prosthetic groups. Another group of nitrite reductases, encoded by *nirK*, contain copper centres that form the redox-active part of the protein (Zumft, 1997). *Neisseria gonorrhoeae* encodes a NirK homologue called AniA (Clark *et al.*, 1987; Clark *et al.*, 1988; Householder *et al.*, 1999). AniA functions as a homotrimer of three 48 kDa monomers, each of which contains two copper-binding sites. The type I copper centre is the site where electrons arrive from the respiratory chain. Electrons then pass to the type II copper centre, where nitrite is bound and subsequently reduced by one electron to nitric oxide and water (Boulanger & Murphy, 2002). Whereas most nitrite reductases are periplasmic (Zumft, 1997), AniA is unusual as it is attached to the outer membrane (Boulanger & Murphy, 2002).

AniA is the most highly upregulated protein in the gonococcus under anaerobic conditions (Clark *et al.*, 1987; Clark *et al.*, 1988). The expression of *aniA* is induced by the two-component NarQP regulator and the oxygen-responsive regulator, FNR (regulator of Fumarate and Nitrate Reduction) (Lissenden *et al.*, 2000; Overton *et al.*, 2006). NarQ is the sensor kinase component of the two-component NarQP system, and NarP is the response regulator. NarP binds upstream of the AniA promoter and activates the transcription of *aniA* in response to a signal from NarQ. Unlike the NarQ protein from *Escherichia coli*, which is

activated by nitrate, the gonococcal NarQ protein is ligand-insensitive and exists in a constantly activated form (Overton *et al.*, 2006). FNR is a transcription factor that is active when oxygen levels are low. Under these conditions it functions as a dimer, where each monomer contains a [4Fe-4S] iron-sulphur cluster that promotes dimerization of the protein. This allows FNR to bind to promoter regions and induce or repress the expression of downstream genes (Lazazzera *et al.*, 1996). FNR is inactivated in the presence of oxygen, as the [4Fe-4S] clusters become unstable. This results in the formation of two Rieske-type [2Fe-2S] clusters and the dissociation of the dimeric complex into two inactive monomers (Lazazzera *et al.*, 1996). The [2Fe-2S] clusters in the FNR monomers are themselves labile to oxygen, resulting in the accumulation of apo-FNR protein under aerobic growth conditions (Sutton *et al.*, 2004a; Sutton *et al.*, 2004b). FNR induces the expression of *aniA* when gonococci are grown under oxygen-limited conditions (Lissenden *et al.*, 2000). NsrR (Nitric oxide-Sensing Repressor) is a transcription factor that represses the expression of downstream genes by binding to their promoter regions and preventing transcription. The ligand of NsrR is nitric oxide, the binding of which results in the release of the NsrR protein from promoter DNA and allows the transcription of genes to proceed (Overton *et al.*, 2006). Under oxygen-limited conditions, the expression of *aniA* is activated by FNR to a low level, but this expression is increased further in the presence of nitric oxide, which binds to NsrR and displaces it from the *aniA* promoter. In this fashion the expression of *aniA* is increased. When levels of nitric oxide rise above a certain level, FNR becomes deactivated and *aniA* expression levels decrease. This feedback mechanism prevents the expression of more AniA protein and therefore the production of more nitric oxide from nitrite. This protects the cell from potential damage by excess levels of nitric oxide (Householder *et al.*, 1999; Overton *et*

al., 2006; Heurlier *et al.*, 2008).

The nitric oxide produced from both the activity of AniA and host cell defence systems must be removed in order to avoid damage to the gonococcus. The nitric oxide reductase (NorB), found in the cytoplasmic membrane, reduces nitric oxide to nitrous oxide (Householder *et al.*, 2000). The catalytic subunit of nitric oxide reductases is NorB, which contains a *b*-type heme. It is often paired with the NorC subunit, which contains a membrane-bound *c*-type heme that acts as the electron donor to NorB. They form a cytochrome *bc*-type complex and are termed cNORs (Berks *et al.*, 1995). Alternatively, some nitric oxide reductases, such as the gonococcal NorB, are quinol-dependent as they receive their electrons directly from quinol rather than from a *c*-type cytochrome. These are termed qNORs and consist solely of a NorB subunit (de Vries & Schroder, 2002). Although the quinol-dependent NorBs encode an *N*-terminal domain homologous to NorC, this domain lacks cytochrome *c* (Hendriks *et al.*, 2000). NorB is similar to subunit I of the HCO family, although their catalytic centres differ, containing iron and copper, respectively (Van Der Oost *et al.*, 1994). Although NorB is not a proton pump and does not directly contribute to proton motive force (Hendriks *et al.*, 2002), the reduction of nitric oxide by NorB requires electrons. These electrons originate from ubiquinol, which receives electrons from the proton-pumping NADH dehydrogenase. Therefore, the reduction of nitric oxide can be considered to be energy conserving, albeit indirectly.

Like AniA, the expression of NorB is repressed by NsrR and therefore activated in the presence of nitric oxide (Overton *et al.*, 2006). However, unlike AniA, the expression of NorB

is not dependent on FNR, so nitrite reduction and nitric oxide reduction processes are not completely coordinated. This is because nitric oxide reduction might be required when nitrite is not present or when oxygen levels are high and FNR is inactivated, i.e. when nitric oxide is produced by external sources such as the host immune system (Overton *et al.*, 2006). Another NsrR-repressed gene is *dnrN*, the protein product of which repairs damage to Fe-S centres of proteins caused in part by nitric oxide. The expression of *dnrN* is de-repressed by the binding of nitric oxide to NsrR, allowing DnrN to be synthesised in response to the presence of NO (Rodionov *et al.*, 2005; Overton *et al.*, 2008).

Many members of the *Neisseria* genus have the *nosRZDFYL* gene cluster, which encodes a nitrous oxide reductase (Barth *et al.*, 2009). Gene clusters coding the nitrous oxide reductase have been found in the pathogenic *Neisseria*, but no nitrous oxide reductases are functional in either of these species. In the case of the gonococcus, the genes are expressed, but the proteins are non-functional due to nonsense mutations in three genes (Cardinale & Clark, 2005). The meningococcus has a deletion in the gene cluster, meaning that it lacks the catalytic subunit, NosZ, and is therefore also unable to reduce nitrous oxide to dinitrogen (Barth *et al.*, 2009). This implies that the gonococcus has adapted to its environment where such an enzyme is not essential for survival, resulting in genome reduction. The lack of nitrate reductases and nitrous oxide reductases therefore results in a truncated denitrification pathway in the gonococcus (Overton *et al.*, 2006).

1.7 Electron transfer chains to nitrite

In many organisms, nitrite is used as an alternative electron acceptor to oxygen. The nature

of electron donors to nitrite reductases varies between different organisms, but they are often *c*-type cytochromes or blue-copper proteins known as azurins. The cd_1-type nitrite reductase of *Paracoccus denitrificans* receives electrons from two sources, namely cytochrome c_{550} and pseudoazurin (Paz) (Moir & Ferguson, 1994; Koutny *et al.*, 1999; Pearson *et al.*, 2003). Although *Pseudomonas aeruginosa* also encodes a cd_1-type nitrite reductase, it instead receives electrons from two *c*-type cytochromes, NirM and NirC (Hasegawa *et al.*, 2001). There are few published reports of the identity of the full complement of electron donors to copper-containing nitrite reductases. In *Bradyrhizobium japonicum*, cytochrome c_{550} is believed to be the only electron donor to NirK (Bueno *et al.*, 2008). However, in *Rhodobacter sphaeroides*, although cytochromes c_2 and c_y have been implicated in electron donation to the copper-containing nitrite reductase, it has been suggested that an additional electron donor exists (Laratta *et al.*, 2006). Interestingly, the azurin of *Pseudomonas aureofaciens* donates electrons to a NirK orthologue (Zumft *et al.*, 1987). Another azurin-like protein, halocyanin, is found in *Natronobacterium pharaonis* and is hypothesised to be a mobile electron donor, perhaps to NirK orthologues found in other haloarchaea such as *Haloarcula marismortui* and *Haloferax denitrificans* (Scharf & Engelhard, 1993; Bonete *et al.*, 2008).

It is likely that the gonococcal nitrite reductase, AniA, receives electrons from either *c*-type cytochromes or azurins. Like cytochromes, azurins can be oxidised and reduced, but rather than containing an iron atom as their redox-active centre, azurins contain a copper atom. Azurin was first isolated from *Pseudomonas aeruginosa* in 1958, where its ability to be oxidised (to produce a blue colour) and reduced raised the possibility that it could function

as an electron carrier (Horio, 1958). It was later found to donate electrons to cytochrome c_{551} (Horio *et al.*, 1960; Ambler & Tobari, 1985). The gonococcus encodes a lipid-modified azurin, Laz (NG0994), which is attached to the outer membrane (Woods *et al.*, 1989; Trees & Spinola, 1990). Laz could play a role in gonococcal electron transfer from ubiquinol to terminal electron acceptors, such as AniA. Furthermore, if the midpoint potential of Laz is comparable to or greater than the midpoint potential of cytochrome c_1, it might be possible for Laz to receive electrons from the cytochrome bc_1 complex as well as from ubiquinol. A gonococcal *laz* mutant is sensitive to hydrogen peroxide (Wu *et al.*, 2005), so it is also possible that Laz is an electron donor to the cytochrome *c* peroxidase, CCP.

1.8 *c*-type cytochromes as redox carriers

Cytochromes are redox proteins that contain heme as their prosthetic group. The central iron atom is the redox-active part of the molecule, and it can be reduced by one electron, thus changing the redox state of the iron atom from +3 to +2. Unlike other types of cytochromes, such as *b*-type, *o*-type, *a*-type and *d*-type, *c*-type cytochromes are the only type to bind their heme group covalently via a CXXCH amino acid motif (Figure 1.5). The thiol groups of the cysteine residues in this motif bind to the vinyl groups of the heme molecule, and the iron atom is axially liganded to the histidine residue (Figure 1.5). As *c*-type cytochromes are always found in the periplasm, it was thought for many years that this covalent binding prevented the heme molecule from becoming dissociated from the protein and being lost to the environment (Wood, 1983). However, since then other types of cytochrome such as *b*- type cytochromes have been found to reside in the periplasm (Barker & Ferguson, 1999). It has been suggested that the covalent attachment of heme allows more

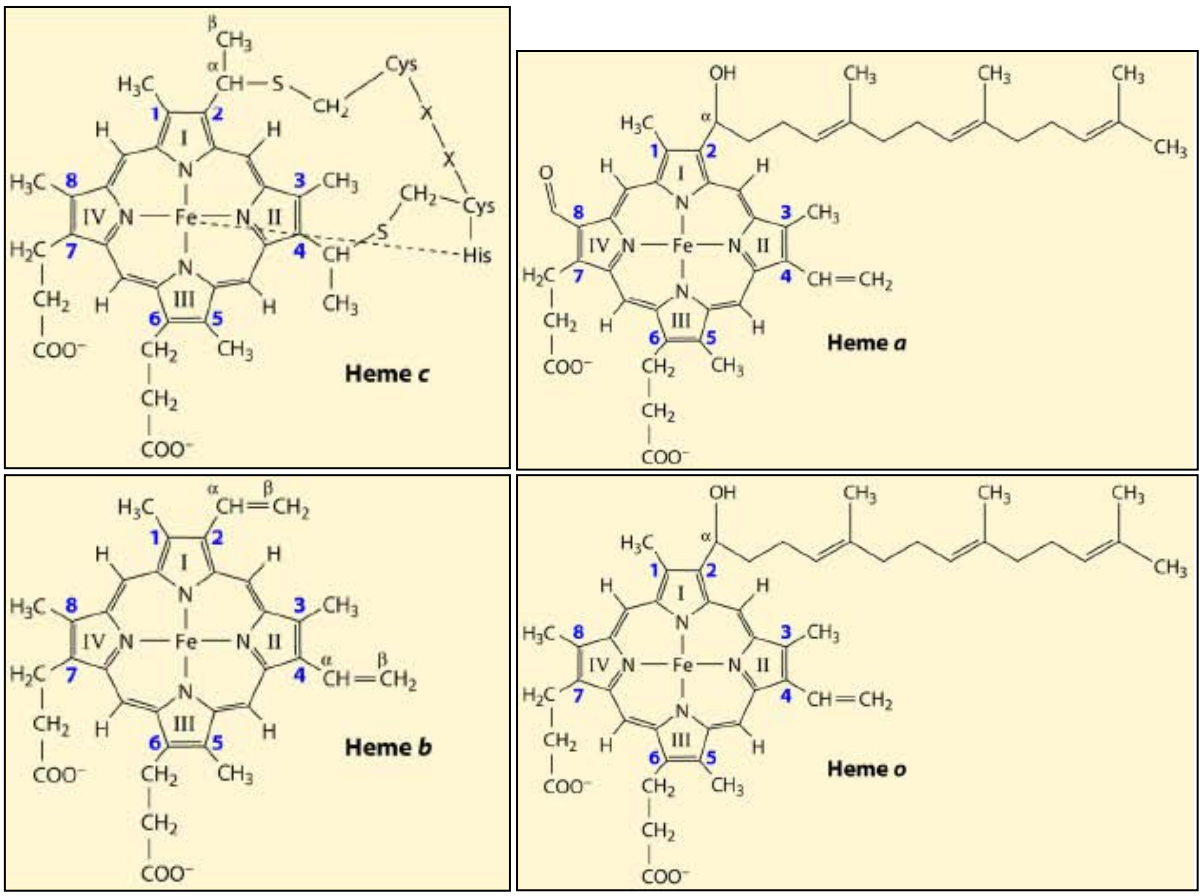

Figure 1.5 – Structure of hemes *c*, *a*, *b* and *o*

Image adapted from Kranz *et al*., 2009.

efficient electron transfer between the redox centres of *c*-type cytochromes containing multiple clustered heme groups. However, the existence of *c*-type cytochromes containing only one heme group might preclude this possibility (Allen *et al.*, 2003). Whatever the reason for the covalent attachment of heme in *c*-type cytochromes, the assembly of the holo-proteins is complex and involves multiple components. Apo-cytochrome *c* is exported from the cytoplasm to the periplasm using the Sec system (Mori & Ito, 2001). As the attachment of heme to the protein is independent from the Sec pathway, bacteria must covalently attach heme to the apo-cytochrome *c* using one of two biogenesis pathways, named Systems I and II. Many α- and γ-proteobacteria use system I, which consists of eight proteins encoded by the *ccm*ABCDEFGH genes (Grove *et al.*, 1996; Allen *et al.*, 2003). System II, which consists of 3 or 4 proteins, is used by most β- and some ε-proteobacteria, as well as Gram positive bacteria and cyanobacteria (Kranz *et al.*, 2002). In the case of the *Neisseria* genus, System II is used. The CcsA and CcsB proteins (annotated as ResC and ResB in the *N. gonorrhoeae* FA1090 genome, respectively) form a complex and export heme from the cytoplasm to the periplasm (Figure 1.6) (Ahuja *et al.*, 2009; Kranz *et al.*, 2009). The CcsA protein then aligns the heme molecule to allow its covalent attachment to the apo-cytochrome *c*. The cysteine groups in the CXXCH amino acid motif of the protein are reduced by the general thioredoxin DsbD (Kumar *et al.*, 2011), as well as the specific thioredoxin CcsX. The heme group held by the CcsA protein can then be covalently attached to the CXXCH amino acid motif in the apo-cytochrome *c*, thus producing holo-cytochrome *c*.

Some bacteria are able to use *c*-type cytochromes to transfer electrons to extracellular electron acceptors, allowing them to use dissimilatory metal reduction (Shi *et al.*, 2009).

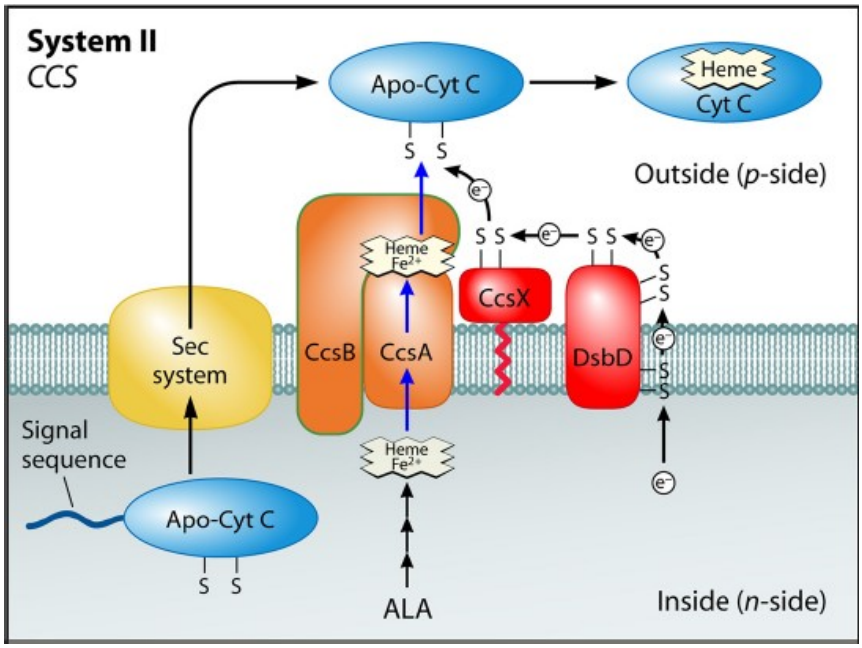

Figure 1.6 – Cytochrome *c* biogenesis

Cytochrome *c* biogenesis by System II. Proteins in orange represent components that export heme from the cytoplasm to the periplasm. Proteins in red are those involved in the reduction of the apo-cytochrome *c*. ALA represents δ-aminolevulinic acid, a precursor of heme biosynthesis. Image from Kranz *et al*., 2009.

Oxidised metals such as iron or magnesium oxides are not easily soluble in water, and therefore bacteria reduce these compounds extracellularly. In one such example, some *Geobacter* species such as *Geobacter sulfurreducens* are able to reduce metal oxides via their type IV pili and *c*-type cytochromes OmcE, OmcS and PgcA. It is hypothesised that OmcE and OmcS, which are extracellular *c*-type cytochromes, receive electrons from the inner membrane, which they then transfer to their type IV pili (Mehta *et al.*, 2005; Reguera *et al.*, 2005; Tremblay *et al.*, 2011). The protrusive nature of pili would therefore allow the bacteria to reduce metal oxides at a distance from their outer membrane.

1.9 The eight *c*-type cytochromes of *Neisseria gonorrhoeae*

The gonococcus is a prolific source of *c*-type cytochromes and has eight in total (Winter & Morse, 1975; Turner *et al.*, 2005). Three of these *c*-type cytochromes are cytoplasmic membrane proteins: cytochrome c_1 from the bc_1 complex (NG2031), and CcoO (NG1373) and CcoP (NG1371) from the cytochrome cbb_3 complex. A further two *c*-type cytochromes are outer membrane proteins, namely cytochrome *c* peroxidase (NG1769) and cytochrome *c*' (NG1080), both of which have been previously discussed. The remaining three cytochromes, cytochromes c_2, c_4 and c_5 (encoded by *cccA*, *cycA* and *cycB* genes, respectively), are believed to act as electron shuttles, passing electrons to the terminal reductases and other proteins involved in protection against reactive oxygen and nitrogen species.

Cytochromes c_4 are dihemic proteins of about 200 residues in length. They are found in all β-proteobacteria, in some γ-proteobacteria such as *Azotobacter* and *Pseudomonas*, and in some instances can be found in α-proteobacteria such as *Bradyrhizobium* and *Rhodobacter*

(Bertini *et al.*, 2006). The gonococcal cytochrome c_4 (NG0101) is made up of 206 residues with a molecular weight of 22 kDa. Although the region between residues 4 and 16 is highly hydrophobic, suggesting the presence of a transmembrane domain, residues 1 to 18 encode a cleavable N-terminal sequence, so cytochrome c_4 might not be attached to the cytoplasmic membrane. The role of cytochrome c_4 in other bacteria is usually as an electron donor. For example, the *A. vinelandii* cytochrome c_4, which is 36% identical to the cytochrome c_4 of *Neisseria gonorrhoeae* (Figure 1.7), exists as part of a bifurcated pathway between the cytochrome bc_1 complex and the cbb_3-type oxidase (Ng *et al.*, 1995). The gonococcal cytochrome c_4 is 99.5% identical to the cytochrome c_4 of *Neisseria meningitidis* and 33% identical to the cytochrome c_4 of *Vibrio cholerae* (Figure 1.7). In the case of *V. cholerae*, cytochrome c_4 is an electron donor to the cbb_3-type oxidase (Chang *et al.*, 2010). There has been a report that the cytochrome c_4 of *Legionella pneumophila*, which is 34% identical to the gonococcal cytochrome c_4 (Figure 1.7), is essential for siderophore expression (Yip *et al.*, 2011). Therefore, although most cytochromes c_4 seem to be electron donors to cbb_3-type oxidases, this might not always be their sole function.

Gonococcal cytochrome c_5 (NG1328) is a 29 kDa protein made up of 279 residues. Residues 16-32 are mostly hydrophobic, suggesting that the gonococcal cytochrome c_5 could be tethered to the cytoplasmic membrane by a transmembrane domain. The nature of cytochromes c_5 depends on the class of proteobacteria from which they originate. Species in most classes of proteobacteria have monohemic cytochromes c_5; however, species that fall in the β-proteobacteria class, such as *Neisseria gonorrhoeae*, generally have dihemic cytochromes c_5. The gonococcal cytochrome c_5 is therefore most similar to cytochromes c_5

Figure 1.7 – Multiple alignment of cytochromes c_4

Multiple alignment of cytochromes c_4 from *Neisseria gonorrhoeae* FA1090 (labelled Ng), *Azotobacter vinelandii* (labelled Av), *Vibrio cholerae* CIRS101 (labelled Vc) and *Legionella pneumophila* (labelled Lp). Heme-binding motifs are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

found in other β-proteobacteria; for example, it is 53% similar to cytochromes c_5 found in *Chromobacterium violaceum* ATCC12472 and *Thiobacillus denitrificans* ATCC25259, and 49% similar to *Ralstonia eutropha* H16 cytochrome c_{555} (Figure 1.8). Cytochrome c_5 is most similar to the cytochrome c_5 of *Neisseria meningitidis*, but differs from the monohemic cytochromes c_5 of γ-proteobacteria. However, domain 1 of the gonococcal cytochrome c_5 is 53% and 49% similar to the whole of the cytochromes c_5 of *V. cholerae* 1587 and *A. vinelandii* (Figure 1.9), respectively, suggesting that perhaps domain 1 of the gonococcal cytochrome c_5 might have a similar role to these other cytochromes c_5. In *V. cholerae*, it has been suggested that cytochrome c_5 is a poor electron donor to the cbb_3-type oxidase, so its function in this bacterium remains unknown (Chang *et al.*, 2010). However, in *A. vinelandii*, the monohemic cytochrome c_5 and dihemic cytochrome c_4 form a bifurcated pathway of electron transfer from the cytochrome bc_1 complex to the cbb_3-type terminal oxidase(Wilson & Wilson, 1955; Tissieres, 1956; Jones & Redfearn, 1967; Ng *et al.*, 1995; Rey & Maier, 1997). The deletion of both cytochromes c_4 and c_5 resulted in the loss of activity of the cytochrome *c* terminal oxidase at low oxygen affinities, although this was compensated by the alternative terminal oxidases of the organism (Rey & Maier, 1997). The reason for the presence of two routes from the cytochrome bc_1 complex to the terminal oxidase is currently unknown. In *A. vinelandii* it is possible to construct a double mutant defective in both cytochromes c_4 and c_5 because this bacterium has more than one terminal oxidase, and therefore the loss of both cytochromes c_4 and c_5 was not lethal to the cell. If only one terminal oxidase were available to an organism, it might be impossible to delete its upstream electron donors.

The gonococcal cytochrome c_2 (NG0292) is a monohemic, 16.5 kDa protein of 155 residues

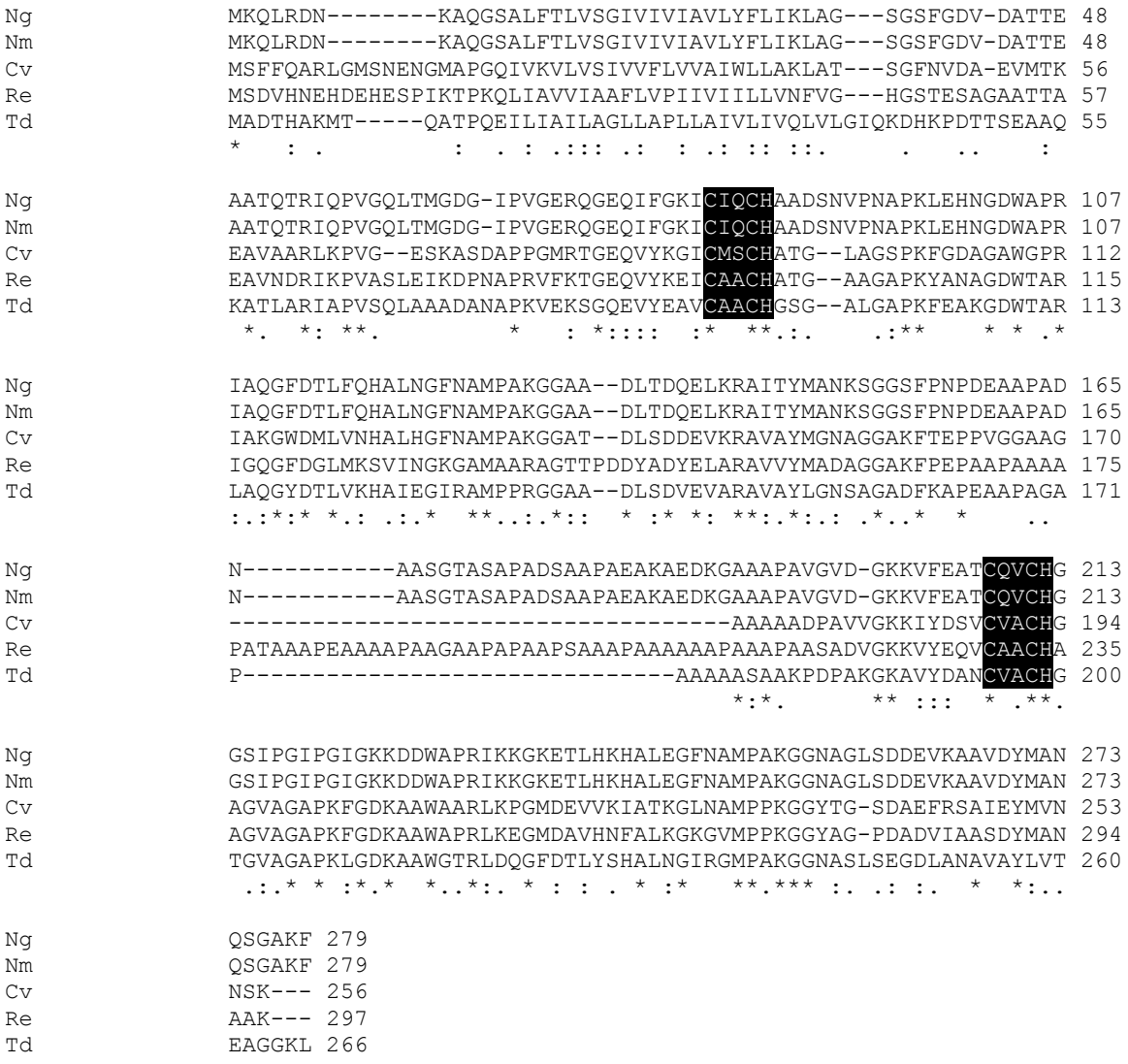

Figure 1.8 – Multiple alignment of cytochromes c_5

Multiple alignment of cytochromes c_5 from *Neisseria gonorrhoeae* FA1090 (labelled Ng), *Chromobacterium violaceum* ATCC12472 (labelled Cv), *Thiobacillus denitrifications* ATCC25259 (labelled Td), *Ralstonia eutropha* H16 (labelled Re) and *Neisseria meningitidis* MC58 (labelled Nm). Heme-binding motifs are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

Figure 1.9 – Multiple alignment of cytochromes c_5 domain 1.

Multiple alignment of cytochromes c_5 domain 1 *Neisseria gonorrhoeae* FA1090 (labelled Ng), *Vibrio cholerae* 1587 (labelled Vc) and *Azotobacter vinelandii* (labelled Av). Heme-binding motifs are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

encoded by the *cccA* gene. It is distinct from the cytochromes c_2 from other organisms, as it is only 26% similar in amino acid sequence to cytochromes c_2 from *Rhodobacter sphaeroides*, *Rhodopseudomonas globiformis* and *Hydrogenobacter thermophilus* c_{552}. Its closest relative is cytochrome c_2 from *Thermus thermophilus*, a member of the phylum Deinococcus thermos, which has been shown to transfer electrons from the bc_1 complex to both caa_3 and ba_3 oxidases (Soulimane *et al.*, 1997; Mooser *et al.*, 2005). Residues 19 to 141 of gonococcal cytochrome c_2 are 51% similar to residues 101-209 of *T. thermophilus* cytochrome c_{552}, although the cytochromes are only 28% similar overall (Figure 1.10). Although the meningococcal cytochrome c_x is 93% identical to the gonococcal cytochrome c_2, outside of the *Neisseria* genus gonococcal cytochrome c_2 is up to 50% identical to part of some copper-containing nitrite reductases (Figure 1.11). This suggests that cytochrome c_2 could be involved in nitrite reduction.

1.10 Previous characterisation of the electron transfer chain of *Neisseria gonorrhoeae*

To date, the respiratory chain of the gonococcus has not been fully characterised. Early spectroscopic analyses correctly implicated the presence of cytochromes *b* and *c*; however, they incorrectly predicted the existence of a cytochrome *o* (Winter & Morse, 1975). A similar conclusion was drawn from experiments in which the effects of various inhibitors, such as antimycin A and potassium cyanide, on gonococcal respiration were studied (Kenimer & Lapp, 1978). Similar research in *Neisseria meningitidis* indicated a branched respiratory chain containing two terminal oxidases, cytochromes *a* and *o* (Yu & Devoe, 1980). The confusion over the number and identity of terminal oxidases was due to the presence of two *b*-type

Figure 1.10 – Pairwise alignment of cytochrome c_2 from *Neisseria gonorrhoeae* FA1090 and cytochrome c_{552} from *Thermus thermophilus*.

Pairwise alignment of cytochrome c_2 from *Neisseria gonorrhoeae* FA1090 (labelled Ng) and cytochrome c_{552} from *Thermus thermophilus* (labelled Tt). Heme-binding motifs are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

```
Phalo      GQRVYEANCMACHQANGEGIPGAFPPLAKSDYLNNNPLLGVNAIIKGLSGPIKVNNVNYNGVMPAMNL 434
Bbac       GKRIYESSCFACHQSNGQGLPGAFPPLAKSDFLKNK-DKAISAVIHGLEGPIKVNGKEYNSVMPAQIL 434
Mcat       GKATYDSNCAACHQPDGKGVPNAFPPLANSDYLNADHARAASIVANGLSGKITVNGNQYESVMPAIAL 465
Psp        GESVYASNCAACHQPNGTGVPNAFPPLAKSDYLNADPNRAIDAIHNGLSGKITVNGKEYNSVMPAVNL 473
Kkin       GETVYKANCLACHGVEGKGVEGAFPPLAESDYLNADYKRGIQAVVKGLSGEITVNGKKFNSVMPAVAL 477
Ngon       GQKVYESNCIACHGKKGEGRGTAFPPLFRSDYIMNKPHVLLHSMVKGINGTIKVNGKTYNGFMPATAI 460
           *:  * :.* ***  .* *   ***** .**::  .       : :*:.* *.**.  ::..***  :

Phalo      NDEDIANVITFVLNNWDNAGGKVSAEQVAKQRK-------------------------- 467
Bbac       SDEDAANVLTYIYSMWGNSKKVVTPADVKAVRAAGKK--------------------- 471
Mcat       SDQQIANVITYTLNSFGNKGGQLSADDVAKAKKNQAKLILKFN--------------- 508
Psp        DDEKTANVVTYILNSWGNKGGEITPEDVAARK-------------------------- 505
Kkin       NDEDAANVLTFVLNSFGNKGGEVKPEEIAAARK-------------------------- 510
Ngon       SDADIAAVATYIMNAFDNGGGSVTEKDVKQAKGKKNQTDKMPSETGNPASDGIQIKPF 518
           .* . * * *:  . :.*    :.  ::   :
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Figure 1.11 – Multiple alignment of *Neisseria gonorrhoeae* FA1090 cytochrome c_2 and *C*-termini of some copper-containing nitrite reductases.

Phalo, Bbac, Mcat, Psp and Kkin refer to the *C*-termini of copper-containing nitrite reductases from *Pseudoalteromonas haloplanktis* TAC125, *Bdellovibrio bacteriovorus* HD100, *Moraxella catarrhalis, Psychrobacter sp.* 1501 and *Kingella kingae* ATCC23330, respectively. Ngon refers to residues 30-132 of cytochrome c_2 from *Neisseria gonorrhoeae* FA1090. Heme-binding motifs are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

hemes within the *cbb*$_3$-type oxidase. As the existence of cytochrome *cbb*$_3$ complexes was not documented until 1994, the aforementioned researchers assumed that the two b-type hemes observed in their spectroscopic experiments corresponded to the presence of a cytochrome *o* (García-Horsman *et al.*, 1994b). However, it has since been clarified from the genome sequences of both *N. gonorrhoeae* and *N. meningitidis* that only a single terminal oxidase of *cbb*$_3$-type is present in both organisms (Parkhill *et al.*, 2000).

Electron transport chains can be partially characterised using myxothiazol, a lipophilic antibiotic isolated from *Myxococcus fulvus* that acts as an inhibitor of the cytochrome *bc*$_1$ complex (Gerth *et al.*, 1980). Myxothiazol binds to the Q_o site on the cytochrome *b* subunit and obstructs the binding of ubiquinol to the Rieske iron-sulphur protein, which is required for electrons to flow from ubiquinol to the cytochrome *bc*$_1$ complex (Figure 1.2). As a result, the reduction of cytochrome *c*$_1$ cannot take place and electron flow from the cytochrome *bc*$_1$ complex to the rest of the respiratory chain is prevented (Von Jagow *et al.*, 1984; Trumpower, 1990). This means that any pathways that receive electrons from the cytochrome *bc*$_1$ complex will be sensitive to myxothiazol, whereas any components that receive electrons directly from ubiquinol will be unaffected by this inhibitor. As a result, myxothiazol can be a useful tool in determining the organisation of electron transfer pathways.

Gonococci are able to use the artificial electron donor N,N,N',N'-tetramethyl-ρ-phenylenediamine (TMPD). TMPD is a dye which, when reduced by ascorbate, readily donates electrons to both bound and unbound cytochromes *c* (Hill & Nicholls, 1980; Chang

et al., 2010). An assay for TMPD oxidation was used to determine the activity of the cytochrome oxidase of *A. vinelandii* (Jurtshuk *et al.*, 1967), which led to the discovery that *Neisseria gonorrhoeae* is able to oxidise TMPD at very high, quantifiable rates (Jurtshuk & Milligan, 1974). An organism that is able to oxidise TMPD is classed as oxidase positive, a trait shared by members of *Azotobacter*, *Neisseria*, *Rhizobium* and *Pseudomonas* species (Kenimer & Lapp, 1978).

In the meningococcus, the cytochrome bc_1 complex receives electrons from ubiquinol, as does the nitric oxide reductase, NorB (Deeudom *et al.*, 2006). The meningococcus and the gonococcus synthesise almost identical electron transport components. However, the meningococcus inhabits a different niche from the gonococcus and has the potential to spread across the blood-brain barrier (Nassif *et al.*, 1999). Consequently, the meningococcal and gonococcal respiratory chains might be organised differently.

1.11 Aims of this project

The first aim of this project was to determine whether cytochromes c_4 and c_5 are both essential for electron transfer to the terminal oxidase, and whether cytochrome c_2 could provide an alternative electron transfer route. Single and double mutants lacking combinations of these three *c*-type cytochromes would be tested for their ability to grow under oxygen-sufficient conditions and to reduce oxygen.

A second aim of this work was to establish which redox proteins are electron donors to the nitrite reductase, AniA. The lipid-associated azurin, Laz, and cytochromes c_4, c_5, and c_2 are

obvious candidates for this role, so deletion mutants lacking combinations of these mutations would be tested for their ability to reduce nitrite. Unlike many similar nitrite reductases, the gonococcal AniA is localised to the outer membrane. It was not known how electrons were able to traverse the periplasm from the inner membrane-bound redox proteins to AniA. It was therefore possible that novel electron donors might exist that would allow sufficient electrons to reach AniA. Consequently, a third aim of this project was to identify any novel electron transfer pathways to AniA that might exist in the gonococcus.

The function of cytochrome c_2 is unknown as there are no obvious homologues of this protein in other bacteria. However, the fact that it is a *c*-type cytochrome suggested that it might be an electron donor to either the terminal oxidase or the nitrite reductase. Prior to the current work, the gonococcal cytochrome c_2 had never been visualised on an SDS-PAGE gel, although RT-PCR experiments had proven that the cytochrome c_2 gene, *cccA*, is transcribed (Li, 2009). Its role in electron transfer remained to be elucidated, so a fourth aim of this work was to characterise this *c*-type cytochrome and determine its role, if any, in the electron transfer chain of the gonococcus.

The final aim of this project was to understand how the arrangement of electron transfer pathways in the gonococcus allows the bacterium to reduce available terminal electron acceptors efficiently and thereby harness energy for growth.

Chapter 2

Materials and Methods

2.1 Bacterial strains

Bacterial strains used in this project are shown in Table 2.1.

2.2 Media and media supplements

Gonococci were cultured on GC agar plates. One litre of GC agar (GCA) contained: 36 g of GC medium base (Appleton Woods); 6 g of bacteriological agar; and 10 mL of Kellogg's supplement. Kellogg's supplement contained (per 200 mL): 8.4 g of $NaHCO_3$; 18 g of glucose; 2 g of L-glutamate; 0.004 g of co-carboxylase; and 0.1 g of $Fe(NO_3)_3$. Kellogg's supplement was sterilised by filtration through a 0.2 µm filter, divided into 10 mL aliquots and stored at -20°C until required. GCA without Kellogg's supplement was autoclaved, and the Kellogg's supplement added aseptically just before plates were poured. Where required, gonococcal agar plates were also supplemented with 3 mM isoleucine to induce piliation in preparation for transformation experiments (Larribe *et al.*, 1997). GCA plates were stored at 4°C for no longer than a week. For liquid cultures, gonococci were cultured in GC broth (GCB). One litre of GCB contained: 15 g of protease peptone #3 (Appleton Woods); 4 g of K_2HPO_4; 1 g of KH_2PO_4; 1 g of soluble starch; 5 g of NaCl; and 10 mL of Kellogg's supplement. Again, Kellogg's supplement was added aseptically after autoclaving. GCB was stored in an incubator at 37°C for no longer than a week.

2.3 Growth of *Neisseria gonorrhoeae*

Growth of *Neisseria gonorrhoeae* involved three main stages: plate growth; pre-culture; and culturing stages. Bacterial suspensions of gonococci stocks were kept in liquid nitrogen

Table 2.1 – Bacterial strains used in this work

Strain	*Genotype/Characteristics*	*Source*
***E. coli* strains**		
JM109	*recA1, endA1, gyrA96, thi, hsdR17* (r_k^-,m_k^+), *relA1, supE44, Δ*(*lac-proAB*), [F',*traD36, proAB, lacI*^{q}*ZΔM15]*	Promega
XL1-Blue supercompetent	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*ZΔM15* Tn*10* (Tet^R)]	Stratagene
RV	Δ*lac*	Laboratory stocks
***Neisseria gonorrhoeae* strains**		
F62	Parental strain	Laboratory stocks
AHGC100	*kan*^{R}	This work
AHGC101	*ccoP* C368A *kan*^{R}	This work
AHGC102	*ccoP* C371A *kan*^{R}	This work
AHGC103	*ccoP* C371A H372A *kan*^{R}	This work
AHGC104	*ccoP* C368A C371A H372A *kan*^{R}	This work
JCGC10	*ΔaspC-lctP::placI-cycA*$^+$ *ermC*	This work
JCGC11	*ΔaspC-lctP::placI-cycB*$^+$ *ermC*	This work
JCGC100	*fnr::ermC*	(Lissenden *et al.*, 2000)
JCGC140	*ΔaspC-lctP::placI-cccA*$^+$ 14 bp upstream, *ermC*	This Work
JCGC142	*cccA::cat ΔaspC-lctP::placI-cccA*$^+$ 14 bp upstream, *ermC*	This Work
JCGC150	*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream, *ermC*	This Work
JCGC151	*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream *cccA* T-22A T-21A C-20G, *ermC*	This Work
JCGC152	*cccA::kan*^{R} *ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream, *ermC*	This Work
JCGC153	*cccA::kan*^{R} *ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream *cccA* T-22A T-21A C-20G, *ermC*	This Work
JCGC200	*aniA::kan*^{R}	Laboratory stocks
JCGC214	*cycP::ermC*	Laboratory stocks
JCGC216	*cycP::ermC cccA::cat*	This work
JCGC310	*cycB Δ*200-279*::ermC*	This work
JCGC311	*cycB Δ*200-279*::cat*	This work
JCGC315	*cycB Δ*200-279*::ermC cccA::cat*	This work
JCGC317	*cycB Δ*200-279*::ermC laz::cat*	This work
JCGC320	*ccoP* C368A *kan*^{R} *cycB Δ*200-279*::ermC*	This work
JCGC321	*ccoP* C368A *kan*^{R} *cycB Δ*200-279*::cat*	This work
JCGC330	*ccoP* C368A *kan*^{R} *cccA::cat*	This work
JCGC335	*ccoP* C368A *kan*^{R} *laz::cat*	This work
JCGC340	*ccoP* C368A *kan*^{R} *cycA::ermC*	This work
JCGC350	*ccoP* C368A *kan*^{R} *cycB Δ*200-279*::ermC cccA::cat*	This work
JCGC351	*ccoP* C368A *kan*^{R} *cycA::ermC cccA::cat*	This work

Strain	***Genotype/Characteristics***	***Source***
JCGC352	*ccoP* C368A *kan*^{R} *cycB Δ*200-279*::ermC laz::cat*	This work
JCGC355	*ccoP* C368A *kan*^{R} *cycP::ermC cycB Δ*200-279*::cat*	This work
JCGC360	*ccoP* C368A *kan*^{R} *cycP::ermC*	This work
JCGC800	*cycA::ermC*	Laboratory stocks
JCGC801	*cycA::kan*^{R}	Laboratory stocks
JCGC805	*cycA::*3xFLAG *kan*^{R}	Laboratory stocks
JCGC807	*cccA::*3xFLAG *kan*^{R}	This work
JCGC820	*cycA::kan*^{R} *ΔaspC-lctP::placI-cycA*$^{+}$ *ermC*	This work
JCGC850	*cycB::ermC*	Laboratory stocks
JCGC851	*cccA::kan*^{R}	Laboratory stocks
JCGC852	*cycB::ermC cccA::kan*^{R}	Laboratory stocks
JCGC853	*cycA::ermC cccA::kan*^{R}	Laboratory stocks
JCGC860	*cycB::cat*	This work
JCGC861	*cccA::cat*	This work
JCGC870	*cycB::cat ΔaspC-lctP::placI-cycB*$^{+}$ *ermC*	This work
JCGC880	*cycA::kan*^{R} *cycB::cat ΔaspC-lctP::placI-cycB*$^{+}$ *ermC*	This work
JCGC890	*laz::kan*^{R}	Laboratory stocks
JCGC891	*laz::ermC*	Laboratory stocks
JCGC892	*laz::ermC cccA::kan*^{R}	Laboratory stocks
JCGC893	*laz::cat*	This work

storage or at -80°C after snap-freezing in liquid nitrogen. Each time a particular strain was required for plate growth, 2 µL of the suspension was spread across a GCA plate using a wire loop. Plates were incubated for 24 h at 37°C in a candle jar containing a moistened paper towel, which provided both oxygen-limited and humid growth conditions. After 24 h, disposable sterile swabs were used to create a lawn of bacteria onto multiple GCA plates, the number of plates varying according to the number of cultures required for each experiment. Plates were incubated for a further 20 h at 37°C in a candle jar. After 20 h incubation, each plate was swabbed into a glass universal bottle containing 10 mL of pre-warmed GCB, which was pre-supplemented with 1% (v/v) Kellogg's supplement. These liquid pre-cultures were grown at 37°C in an orbital shaking water bath at 100 rpm for one hour to allow gonococci time to adapt from plate growth to growth in liquid culture.

After 1 hour, the 10 mL pre-cultures were added to 100 mL conical flasks containing varying levels of GCB, which was pre-supplemented with 1% (v/v) Kellogg's supplement. The volume of liquid within the conical flasks was 20 mL for aerobic growth conditions and 50 mL for oxygen-limited conditions, making the total volume of culture 30 mL and 60 mL, respectively. The volume of pre-culture added to the conical flasks was adjusted to ensure that the starting $OD_{650\ nm}$ of the cultures were comparable. Cultures grown under oxygen-limited conditions were supplemented with 5 mM sodium nitrite in order to provide an alternative electron acceptor to oxygen and to stimulate the expression of AniA. Sodium nitrite was added in concentrations of 1 mM and 4 mM $NaNO_2$ after 1 h and 2 h of growth, respectively. The purpose of the addition of a primary low concentration of sodium nitrite was to allow the induction of expression of the nitrite reductase, AniA, to take place before

cultures were exposed to higher, potentially more toxic levels of nitrite. Samples of each culture (1 mL) were taken at regular intervals, and the $OD_{650\ nm}$ of these samples was measured using a spectrophotometer.

2.4 Harvesting of cultures

Cultures of bacteria were harvested at different optical densities depending on the assay for which they were required. Cultures were harvested by centrifugation at 9,000 rpm for 1 minute at 4°C using a Beckmann MSE-18 centrifuge. The resulting pellet was re-suspended in 2 mL of cold, pH 7.4, 50 mM potassium phosphate buffer (which contained, per litre: 7.26 g K_2HPO_4 and 1.13 g KH_2PO_4) and homogenised with a cold hand-held homogeniser. The resulting solution was aliquoted into 1.5 mL Eppendorf tubes and centrifuged at 13,000 rpm for 1 minute at room temperature. The pellet was then resuspended in up to 900 µL of the same phosphate buffer and the optical density of cells calculated by measuring the $OD_{650\ nm}$ of a 100-fold dilution of the cell solution. For both oxygen and nitrite reduction assays, washed whole cells with an $OD_{650\ nm}$ of between 20 and 40 were used. The quantity of cells was then calculated using the estimation of an $OD_{650\ nm}$ of 1 being equivalent to 0.4 g of bacteria per litre.

2.5 Gram's staining

The technique of Gram's staining was employed to ascertain that cultures of *Neisseria gonorrhoeae* were not contaminated with other micro-organisms. A 1 mL sample of the culture was poured into a 1.5 mL Eppendorf tube and centrifuged at 13,000 rpm for 1

minute. The resulting pellet was smeared onto a glass slide using a toothpick and fixed by quickly passing the slide through a Bunsen burner flame. Crystal violet was applied to the slide for one minute and then washed off using distilled water. Next, Gram's iodine was applied, left for one minute and washed off using 100% ethanol followed by distilled water. Safranine was then applied for one minute, then washed off with distilled water. The slide was left to dry for a few minutes, after which ceder oil was applied and the slide examined under an optical microscope, usually with 100 x magnification.

2.6 Oxygen reduction assays

An oxygen electrode was used to measure rates of oxygen reduction. Cultures with a total volume of 30 mL were grown as described previously until they reached an OD_{650nm} of between 0.8 and 1, at which point they were harvested. An S1/MINI Clark-type oxygen electrode (Hansatech Instruments) was used in conjunction with the Oxytherm control unit and the Oxygraph Plus program to measure oxygen reduction rates. The electrode had a total volume of 2 mL, and 100 µL of cell suspension was used for each assay. The final concentrations of electron donors in the assay were 50 mM lactate or 5 mM ascorbate with 1 mM N,N,N*,N*,-tetramethyl-ρ-phenylenediamine (TMPD). Where appropriate, a concentration of 1 µM myxothiazol was used. The rest of the chamber contained up to 1.9 mL of 50 mM potassium phosphate buffer (pH 7.4). To calculate the rate of oxygen reduction in nmol. min^{-1}. (mg dry cell mass)$^{-1}$, the following formula was used:

$$\text{Rate} = \frac{\text{gradient calculated by Oxygraph x total vol. (µL)}}{OD_{650\,nm} \text{ cells x } 0.4 \text{ x vol. cells (µL)}}$$

where gradient = $\frac{\Delta\ O_2 \text{ concentration } (\mu M)}{\Delta \text{ time (min)}}$

total vol. = total volume of chamber = 2000 µL

$OD_{650\ nm}$ cells = $OD_{650\ nm}$ of 1/100 dilution of cells x 100

0.4 = dry weight of cells for a culture with $OD_{650\ nm}$ of 1.0 (g per litre)

vol. cells = volume of cells added to chamber (µL)

2.7 Spot testing

In order to spot test for the presence of nitrite in cultures, Griess reagents were used (Griess, 1879). Griess reagent 1 (acidified sulphanilamide) reacts with nitrite to form a diazonium salt (Figure 2.1). When this salt is combined with Griess reagent 2 (N-1 napthylethylene diamine dihydrochloride), an azo chromophore is produced, which absorbs light at a wavelength of 540 nm (Figure 2.1). In this way the presence of nitrite in cultures can be detected. A 1 mL sample of culture was centrifuged in a 1.5 mL Eppendorf tube at 13,000 rpm for one minute. A 40 µL sample of the supernatant was applied to a spot-testing plate. Two drops of 1% sulphanilamide in 1 M HCl was added, followed by two drops of 0.02% N-1 napthylethylene diamine dihydrochloride in distilled water. The presence of nitrite in the culture was indicated by the development of a pink colour.

2.8 Whole cell nitrite reduction assays

The reaction of Griess reagents with nitrite results in the formation of an azo chromophore that absorbs UV light at 540 nm. The concentration of azo chromophore in a solution can therefore be measured spectrophotometrically. As the amount of azo chromophore produced by the reaction of Griess reagents with nitrite is proportional to the abundance of nitrite in the solution, the absorbance of solutions at 540 nm can be used to determine the concentration of nitrite within that solution. Consequently, Griess reagents were used to

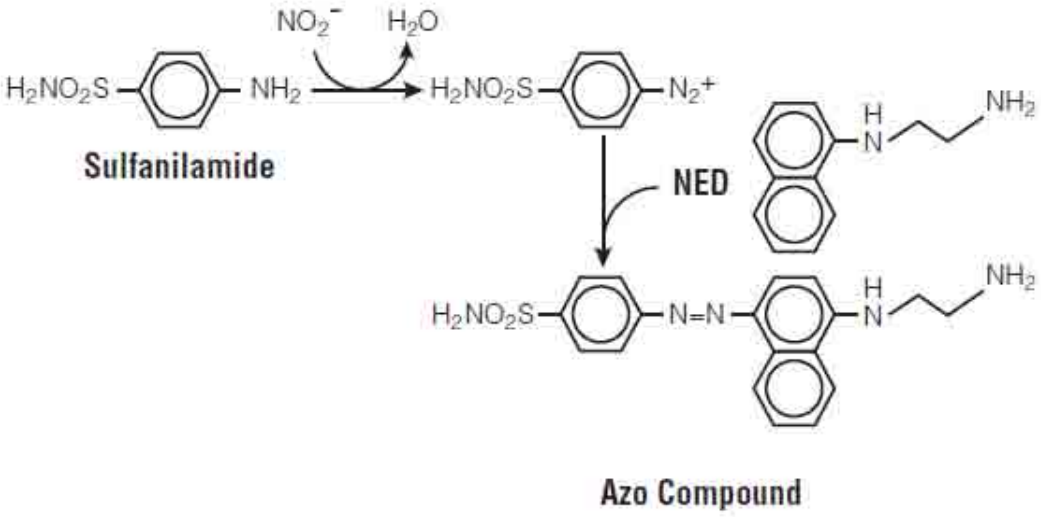


Figure 2.1 – Mechanism of nitrite detection using Griess Reagents

N-1 napthylethylene diamine dihydrochloride is labelled as NED. Modified from Promega Technical Bulletin Griess Reagent System.

measure quantitatively the rate of nitrite reduction of whole, washed cells (Pope & Cole, 1984). Oxygen-limited cultures with a total volume of 60 mL were supplemented with 1 mM and 2 mM sodium nitrite at 1 h and 2 h post-inoculation. Cultures were spot tested until all of the nitrite had been reduced (at an $OD_{650\ nm}$ of between 0.5 and 0.7) to ensure that AniA expression had taken place. Cultures were then harvested as described previously. Three assay tubes were set up, each containing 40 mM lactate, 50-150 µL of cells (depending on the $OD_{650\ nm}$ of cultures) and a volume of 50 mM potassium phosphate buffer to create a total volume of 4950 µL. Where relevant, 1 µM of myxothiazol was added to tubes and the volume of phosphate buffer was adjusted accordingly. Where nitrite reduction rates were very low, the total volume of the tubes was halved and the volume of cells doubled to allow more accurate quantification. The tubes were incubated at 30°C in a water bath for 5 minutes, then 50 µL of 0.1 M sodium nitrite was added to start the reaction. The solution was vortexed to ensure homogeneity of the solution. Every three minutes, a 50 µL sample from each tube was removed and added to another tube containing 3 mL of 1% sulphanilamide in 1 M HCl. The solutions in these tubes were vortexed to ensure homogeneity, then 300 µL of 0.02% N-1 napthylethylene diamine dihydrochloride in distilled water was added. Solutions were vortexed again then poured into a 3 mL cuvette. The pink colour was allowed to develop for 30 minutes, after which the OD_{540} was measured using a spectrophotometer. As a control, three tubes containing 3 mL of 1% sulphanilamide in 1 M HCl and 300 µL of 0.02% N-1 napthylethylene diamine dihydrochloride in distilled water were set up, one with 50 µL of distilled water added as a control and the other two with 30 µL or 60 µL of a 1 mM $NaNO_2$ solution to use as a calibration point for assay readings. The rate of nitrite reduction in nmol. min^{-1}. (mg dry cell mass)$^{-1}$ was calculated using the

following formula:

$$\text{Rate} = \frac{\text{gradient} \times 30 \times 20 \times \text{total vol. } (\mu L)}{OD_{540\ nm}\ \text{standard} \times OD_{650\ nm}\ \text{cells} \times \text{vol. cells } (\mu L) \times 0.4}$$

where gradient = $\frac{\Delta\ OD_{540\ nm}}{\Delta\ \text{time (s)}}$

30 = nmol nitrite standard to calibrate the assay
20 = dilution factor (to convert values /50 µL to /1 mL)
total vol. = total volume of assay tube (µL)
$OD_{540\ nm}$ standard = $OD_{540\ nm}$ of nitrite standard sample
$OD_{650\ nm}$ cells = $OD_{650\ nm}$ of 1/100 dilution of cells x 100
vol. cells = volume of cells added to assay tube (µL)
0.4 = dry weight of cells for a culture with $OD_{650\ nm}$ of 1.0 (g per litre)

2.9 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA molecules based on their size. 1% agarose gels were made using 1 g of agarose (Sigma-Aldrich) dissolved in 100 mL of TBE buffer. TBE buffer consisted of 89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA dissolved in 1 L of double-distilled water. The 1% agarose solution was heated in a microwave until all of the agarose had dissolved, then poured into a mould. DNA samples were mixed with DNA blue sample buffer in a ratio of 5 of DNA solution: 2 of buffer solution. HyperLadder™ I (Bioline) was used to determine the size of DNA fragments and to quantify DNA. The gel was placed into a gel tank containing TBE buffer and 0.5 µg mL^{-1} ethidium bromide, and a voltage of 100 or 120 V was applied for 30 to 40 minutes. DNA was visualised and quantified using the Gel Doc™ XR+ System (Bio-Rad) and the Quantity One® program (Bio-Rad).

2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoreses (SDS-PAGE)

Samples of 1 mL were taken from relevant cultures and their $OD_{650\ nm}$ was recorded. Samples were taken after 5 hours of growth for Western analysis or after 7 hours of growth for the staining of covalently bound heme. Bacteria were collected by centrifugation at 13,000 rpm for 1 minute and the supernatant was removed. The amount of sample loading buffer used to suspend bacterial pellets was proportional to the optical density of the original bacterial cultures from which the pellet was acquired. This allowed the same relative amount of protein from each sample to be loaded onto the SDS-PAGE gel. For every $OD_{650\ nm}$ reading of 0.1, 10 µL of sample buffer was used to resuspend bacterial pellets. To denature the proteins, the suspensions of bacteria in sample buffer were boiled at 100°C for 10 minutes prior to loading. Proteins were resolved by Tris-Tricine SDS-PAGE using a 15% resolving (wt/vol) and a 6% stacking polyacrylamide gel made using the following components: Protogel (30% acrylamide/bisacrylamide), 0.75 M Tris-HCl (pH8.3), 1.25 M Tris-HCl (pH 6.8), 20% SDS, 8% ammonium persulphate, N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and distilled water. Broad-range, pre-stained protein marker (New England Biolabs) was used to determine the molecular masses of proteins in samples. Proteins were separated by gel electrophoresis at 50V overnight where staining of covalently bound heme was required, or at 100-160V for 1 h where Western analysis was to follow. Total protein was detected with 0.02% (wt/vol) Coomassie blue. Protein gels were scanned using the GS-800™ Calibrated Densitometer (Bio-Rad) and the Quantity One® program (Bio-Rad).

2.11 Staining of gels for covalently bound heme

Proteins containing covalently attached heme were detected with heme-dependent peroxidase activity (Thomas *et al.*, 1976). SDS-PAGE gels were incubated in 10% trichloroacetic acid for 30 minutes, then placed in a solution containing 6.3 mM TMBZ (3,3',5,5'-tetramethyl-benzidine) dissolved in 75 mL methanol, and 175 mL of 0.25 M sodium acetate (pH 5.0). This was placed in darkness for 2 h, then 350 µL of hydrogen peroxide was added. Once heme-stained bands had appeared, the gel was placed into a solution containing 75 mL of propan-2-ol and 175 mL of 0.25 M sodium acetate (pH 5.0). The gel was scanned then placed into shrink solution (48% methanol, 2% glycerol) and mounted.

2.12 Analysis of AniA and 3xFLAG tag in bacteria by Western analysis

Proteins separated by SDS-PAGE were transferred electrophoretically using a Bio-Rad Trans-D semi-dry blotter onto a polyvinylidene fluoride (PVDF) membrane (Millipore) at 0.25A for 1 hour in blotting buffer (Nilavongse *et al.*, 2006). The membrane was removed and gently agitated overnight in 50 mL of 5% (w/v) non-fat dry milk (Bio-Rad) in TBS within a sealed bag at 4°C. The blocked membrane was washed three times for 5 minutes each with gentle agitation in TBS-Tween. The membrane was transferred to a plastic bag which contained the primary antibody in 10 mL of 5% (w/v) non-fat dry milk in TBS-Tween and was incubated with gentle agitation for 2 h at room temperature. The primary antibody used to detect AniA was anti-AniA (Rabbit) antibody (from J. Moir and M. Thomson) and was diluted 1: 5000. This antibody was raised from whole, recombinant AniA protein from *Neisseria meningitidis* MC58 (Rock *et al.*, 2007). The primary antibody used to detect FLAG-tagged proteins was

anti-FLAG monoclonal (Mouse) antibody (Sigma-Aldrich) and was diluted 1: 5000. The membrane was transferred to a tray and was washed three times for 5 minutes each with gentle agitation in TBS-Tween. The membrane was transferred to a plastic bag containing either the anti-rabbit or the anti-mouse peroxidase-labelled secondary antibody (ECL Plus Western Blotting Detection Reagents, Amersham) diluted 1:5000 in 10 mL of 5% non-fat dry milk TBS-Tween and incubated with gentle agitation for 1 h at room temperature. The membrane was then transferred to a tray and washed three times for 5 minutes each with gentle agitation in TBS-Tween. The membrane was then treated with 5 mL of Solution A and 5 mL of Solution B from the EZ-ECL kit (Biological Industries, Life Technologies) for 5 minutes with gentle agitation. The membrane was then wrapped in cling film and exposed to Hyperfilm ECL (Amersham) for 1 to 20 seconds and developed on the Xograph.

2.13 Molecular techniques

The polymerase chain reaction (PCR) was used to verify the correct construction of plasmid DNA, to transform *Neisseria gonorrhoeae* cells and to confirm that homologous recombination had occurred in the latter. Unless otherwise stated, a standard PCR protocol was used (Table 2.2). The melting temperature of primers was calculated using the formula 64.9 + (G% + C%) 0.41 – 600/n, where G% and C% are the percentages of guanidine and cytosine bases in the primer DNA sequence and n is the number of DNA bases in the primer. PCR reactions included 1 µL of template DNA (either plasmid or genomic DNA), 2 µL of each PCR primer, 5 µL of double-distilled sterile water and 40 µL of PCR Supermix High Fidelity (Invitrogen). All primers were procured from Alta Bioscience Ltd. (University of Birmingham) and are shown in Table 2.3. PCR products were purified before use using the QIAquick® PCR

Table 2.2 – Standard protocol for PCR reactions

	No. cycles	Temperature	Time
Step 1	1	94°C	4 min
Step 2	30	94°C	30 sec
		T_m°C (where T_m = melting temperature of homology region)	1 min
		68°C	X min (where X = Kb size of fragment to be amplified)
Step 3	1	68°C	10 min
Step 4	1	4°C	∞

Table 2.3 – Primers used in this work

Name	Sequence (5'-3')	Restriction site
YLAniAAUF	CGACCTGTTCGGGGTAAGGCGGGGC	
YLAniACDR	CCCGCCTTCGGCGCATAGCTGCG	
GCFNR3	GGCGGATCCGCGCGGTCAAACTCGGG	
GCFNR4	CGCGGATCCGATTTAGGCGGCTGCCGG	
NTCYC4 FOR	GACCCAATGTGCGCGTACCG	
NTCYC4 REV	GTGTGGGAGATATACGGGATTTACTC	
YLC5AUF	CAGGATCCGCGGCAGACTTGGCTACACGGGGTTG	
YLC5BUR	GTCAACAGCCGCTTTGACCGGTGTAGGCCTCGCCGTC	*AgeI*
YLC5CDR	GACTCGGATCCGTGATGGAGCGCCGG	
YLC5DDF	GCGATGAACCGGTCAAAGCGGCTGTTGAC	*AgeI*
YLC552AUF	GGCAAAATGGGTTACACCGAGCACGCAGG	
YLC552BUR2	GGTGGCGGCAATATCACCGGTCGTAGCGGGTGAAAG	*AgeI*
YLC552DDF2	CTACGACCGGTGATATTGCCGCCACC	*AgeI*
YLC552CDR	CGTGCTTCAGGCGGAGGATTTGGCG	
NTERY FOR	AGAAGACCGGTTAAGAGTGTGTTGATAGTGC	*AgeI*
NTERY REV	CAAATTACCGGTAGGCGCTAGGGACC	*AgeI*
YLKanF	CGTAACCGGTAAAGCCAGTCCGCAG	*AgeI*
YLKanR	CGATACCGGTGGAGGATCATCCAGC	*AgeI*
NCC4F	CTACGTTTAATTAACATTGTTGCGTTATCC	*PacI*
NCC4R	CTACGTTTAATTAACATCGATTCCAACGG	*PacI*
ST_CycPFP3	GGTGGTCGGATCCGGATGAAAACCCAAATCAGCCTTGCCG	*BamHI*
ST_CycPRP1	CTGCCCTACGGTTTGAACAAATCG	
NTAZUFOR	CGGCGGCGGCAAAGTTGGAAGAACGG	
NTAZUREV	GCGCAACAGCTCCGTCCACGCC	
AHpGCC4PacIns	GCTGTGGTATGGCTGTG	
AHCptC5F	CTACGTTTAATTAACCTAACCTGACGGCG	*PacI*
AHCptC5R	CTACGTGGCCGGCCATTCATCCGATCGGGCAG	*FseI*
AHCptC2F	CTACGTTTAATTAACACCCGCTACGCAAAATC	*PacI*
AHCptC2Fb	CTACGTTTAATTAAGGAAACGCCAATGAACAC	*PacI*
AHCptC2R	CTACGTGGCCGGCCACGTTCGGGCGATTTGG	*FseI*
AHC2loopSDM_Fb	CGCTACGCAAAATCAACAACAAGGTAAGAAACATTAAGGAAACGCCAATGAAC	
AHC2loopSDM_Rb	GTTCATTGGCGTTTCCTTAATGTTTCTTACCTTGTTGTTGATTTTGCGTAGCG	
AHpGCC4_F	CCCGCATCAAACAGCTCGG	
AHpGCC4_R	CCATTGTTCGGGCGTAGGG	
AHCatAgeIF	ACTGACCGGTGGCAGGCCATGTCTGCC	*AgeI*
AHCatAgeIR	ACTGACCGGTGCTTACTCCCCATCCCC	*AgeI*
AHKanBspEI_F	CCAGTTTCCGGAGCTATCTGGACAAGG	*Bsp*EI
AHKanBspEI_Rb	ATACTTTCCGGATTCATAGAAGGCGGC	*Bsp*EI
AHccoP_ds_F	GCCTGAGTCCAATATGCCGG	
AHccoP_ds_R2a	CCTATATGCCGTCTGAAAGC	
AHccoP_C368A_F	TGGCAAACAGGTTTATGAAACCGTTGCCGCCGCCTGCCATG	
AHccoP_C368A_R	CATGGCAGGCGGCGGCAACGGTTTCATAAACCTGTTTGCCA	
AHccoP_C371A_F	AAACCGTTTGTGCCGCCGCCCATGGCAATGCAATTCC	
AHccoP_C371A_R	GAATTGCATTGCCATGGGCGGCGGCACAAACGGTTTC	
AHccoP_C371A-H372A _F	TTTATGAAACCGTTTGTGCCGCCGCCGCCGGCAATGCAATTCCAGGTATTCCCC	
AHccoP_C371A-H372A _R	GGAATACCTGGAATTGCATTGCCGGCGGCGGCGGCACAAACGGTTTCATAAACC	
AHccoP_C368A-C371A-	GATGGCAAACAGGTTTATGAAACCGTTGCCGCCGCCGCCGCCGGCAA	

Name	Sequence (5’-3’)	Restriction site
H372A _F	TGCAATTCCAGGTA	
AHccoP_C368A-C371A-	GATGGCAAACAGGTTTATGAAACCGTTGCCGCCGCCGCCGCCGGCAA	
H372A _R	TGCAATTCCAGGTA	
AHccoPKan_check	AGAAGCAGCACAAGCCGC	
AHC5-2ndAUF	GGCAGGATGTGCGTCGCC	
AHC5-2ndBURb	CTGCCCACCGGTACCGTTTATTAGTCAACGCCGACCGCAGG	*AgeI*
AHC5-2ndDDF	ACGGTACCGGTGGGCAGGTATCTGCTCCG	*AgeI*
AHC5-2ndCDR	ACAGGCATCCGGATGCCG	
AHC5-2ndint_F	TCGCCGATGCGCTTGCC	
AHC5-2ndint_R	CGCCCGACATATCGAGCC	
AHC2AUFEcoRI	GTCTAGGAATTCACCGAGCACGCAGG	
AHC2BURKpnI	GTCTAGGGTACCGAAAGGTTTGATTTGAATGCCG	
AHC2CDRNheI	GTCTAGGCTAGCGGCGGAGGATTTGGCG	
AHC2DDFXhoI	GTCTAGCTCGAGCGCGGTACTTTCAGCC	

purification kit according to the manufacturer's instructions. DNA was eluted in 50 µL elution buffer (supplied in kit) or double-distilled water and, where required, stored at -20°C.

The pGEM®-T easy vector system (Promega) was used to clone PCR products into a vector to allow further downstream processing. During PCR, most DNA polymerases add an additional deoxyadenosine molecule to the 3′ end of PCR products. The pGEM®-T easy vector is supplied pre-cut with a 3′ deoxythymidine overhang, allowing the efficient ligation of PCR products into the vector. Where required, resulting plasmids were used to construct plasmids that could be used for the homologous recombination of deletion-insertion mutations onto the gonococcal chromosome. Restriction endonucleases (New England Biolabs) were used to create DNA fragments for insertion into these plasmids, using either PCR products or other plasmid DNA. The DNA fragments produced following digestion were separated by agarose gel electrophoresis and the relevant fragment was extracted and purified using the QIAquick® gel extraction kit (Qiagen). The vector was prepared by restriction digestion and treatment with Antarctic Phosphatase (New England Biolabs) in order to prevent recircularisation of vector DNA. The prepared vector and purified DNA fragments were then ligated together using T4 DNA ligase (New England Biolabs). To increase the efficiency of ligation reactions, an insert:vector molar ratio of 3:1 was used. The following formula was used to determine the appropriate amounts of vector and insert DNA to add to the reaction:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Kits and enzymes described above were used according to the manufacturer's instructions.

Plasmids used and generated during this study are shown in Table 2.4.

2.14 Extraction of genomic DNA from *Neisseria gonorrhoeae*

To extract genomic DNA, *N. gonorrhoeae* was plated as described above and incubated for 24 h at 37°C in a candle jar. Sterile swabs were used to transfer bacteria into 1 mL of sterile, double-distilled water and cells were collected by centrifugation at 13,000 rpm for 1 minute. Genomic DNA was then extracted from bacteria using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare), eluted in 200 µL of elution buffer (supplied with kit) and stored at -20°C.

2.15 Use of *Escherichia coli* strains for cloning experiments

Escherichia coli was grown at 37°C with aeration in 20 mL of Luria–Bertani broth (LB) (20 g tryptone, 10 g yeast extract and 10 g of NaCl per litre of distilled water) in a 150 mL conical flask. *E. coli* strains were stored as glycerol stocks at -80°C. A standard protocol was used to make *E. coli* cells competent for transformation with plasmid DNA. *E. coli* cells were inoculated into 2 mL of LB and incubated overnight with shaking at 37°C. The resulting culture was used to inoculate 20 mL of LB, and cultures were incubated with shaking at 37°C until an $OD_{600\ nm}$ of about 0.5 was reached. The culture was kept on ice for 20 minutes, then transferred to sterile centrifuge tubes. Bacteria were collected by centrifugation at 4000 g for 3 minutes, then resuspended in 3 mL of TFB1 buffer. TFB1 buffer contained 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$ and 15% (v/v) glycerol. After incubation on ice for 90 minutes, bacteria were collected by centrifugation at 4000 g for 3

Table 2.4 – Plasmids used in this work

Plasmid	*Characteristics*	*Source*
pGEM® T Easy	Commercially available vector for cloning PCR products	Promega
pLES940	Source of the *cat* gene	(Silver & Clark, 1995)
pSUB11	Source of the *kan*^{R} gene	(Datsenko & Wanner, 2000)
pAHKanBspEI	*kan*^{R} gene flanked by *Bsp*EI sites in pGEM® T-Easy	This work
pAH100	*ccoP* gene cloned into pGEM® T-Easy	This work
pAH101	pAH100 C368A	This work
pAH102	pAH100 C371	This work
pAH103	pAH100 C371A H372A	This work
pAH104	pAH100 C368A C371A H372A	This work
pAH105	*cat* gene ligated into the *AgeI* site of pYL6	This work
pAH106	*kan*^{R} gene ligated into the *AgeI* site of NT100	This work
pAH110	*kan*^{R} gene ligated into the *Bsp*EI site of pAH100	This work
pAH111	*kan*^{R} gene ligated into the *Bsp*EI site of pAH101	This work
pAH112	*kan*^{R} gene ligated into the *Bsp*EI site of pAH102	This work
pAH113	*kan*^{R} gene ligated into the *Bsp*EI site of pAH103	This work
pAH114	*kan*^{R} gene ligated into the *Bsp*EI site of pAH104	This work
pAH107	*cat* gene ligated into the *AgeI* site of pYL12	This work
pAH130	502 bp of non-coding DNA upstream of *cycB* and 595 bp of *cycB* coding DNA linked by an *AgeI* site to 612 bp of DNA downstream of *cycB* in pGEM® T-Easy	This work
pAH131	*ermC* gene ligated into the *AgeI* site of pAH130	This work
pAH132	*cat* gene ligated into the *AgeI* site of pAH130	This work
pAH140	*laz* gene interrupted by *cat* gene in pGEM® T-Easy	This work
pAHC2FCD	336 bp of downstream DNA of the *cccA* gene ligated between the *XhoI* site and the *NheI* site of pDOC-F	This work
pAHC2F	435 bp of upstream and 465 bp of coding DNA of the *cccA* gene ligated between the *EcoRI* site and the *KpnI* site of pAHC2FCD	This work
pDOC-F	Source of 3xFLAG tag	(Lee *et al.*, 2009)
pGCC4	Neisserial Insertional Complementation System *ermC*, *kan*^{R}, *lctP*, *aspC, lacI*^{Q}*, lacP*	(Mehr & Seifert, 1998) (Mehr *et al.*, 2000) (Stohl *et al.*, 2005)
pGCC4_cycA	pGCC4 containing the *cycA* gene flanked by 39 bp of upstream DNA and 103 bp of downstream DNA inserted into the *PacI* site of pGCC4	This work
pGCC4_cycB	pGCC4 containing the *cycB* gene flanked by 28 bp of upstream DNA and 149 bp of downstream DNA inserted between the *PacI* and *FseI* sites of pGCC4	This work

Plasmid	***Characteristics***	***Source***
pGCC4_cccA	pGCC4 containing the *cccA* gene flanked by 50 bp of upstream DNA and 204 bp of downstream DNA inserted between the *PacI* and *FseI* sites of pGCC4	This work
pGCC4_cccAb	pGCC4 containing the *cccA* gene flanked by 14 bp of upstream DNA and 204 bp of downstream DNA inserted between the *PacI* and *FseI* sites of pGCC4	This work
pGCC4_cccA_loop	pGCC4_cccA with potential stem loop locus mutated at 22 bp, 21 bp and 20 bp upstream of the start codon of the *cccA* coding region	This work
pNT100	Gonococcal *cycA* gene cloned into pGEM® T-Easy	(Li *et al.*, 2010)
pNT101	*ermC* gene flanked by *AgeI* sites in pGEM® T-Easy	(Li *et al.*, 2010)
pNT102	*ermC* gene ligated into the *AgeI* site of pNT100	(Li *et al.*, 2010)
pNT502	*laz* gene interrupted by kan^R gene in pGEM® T-Easy	Laboratory stocks
pYL4	kan^R gene flanked by *AgeI* sites in pGEM® T-Easy	(Li *et al.*, 2010)
pYL6	584 bp of DNA upstream of *cycB* linked by an *AgeI* site to 448 bp of DNA downstream of *cycB* in pGEM® T-Easy	(Li *et al.*, 2010)
pYL7	*ermC* gene ligated into the *AgeI* site of pYL6	(Li *et al.*, 2010)
pYL12	430 bp of DNA upstream and 331 bp of DNA downstream of the *cccA* gene in a continuous sequence containing an *AgeI* restriction site at the end of the upstream DNA ligated into pGEM® T-Easy	(Li *et al.*, 2010)
pYL13	kan^R gene ligated into the *AgeI* site of pYL12	(Li *et al.*, 2010)
pYL14	515 bp of DNA upstream of *aniA* linked by a *Bam*HI site to 516 bp of DNA downstream of *aniA* in pGEM® T-easy	(Li, 2009)
pYL15	kan^R gene ligated into the *Bam*HI site of pYL14	(Li, 2009)

minutes and resuspended in 400 µL of a solution of 0.1 M $CaCl_2$ and 15% (v/v) glycerol. Aliquots of 100 µL were snap-frozen in liquid nitrogen then stored at -80°C until required.

Competent *E. coli* cells were transformed with plasmid DNA using the heat-shock protocol. 50 µL of competent cells were added to between 1 and 5 µL of plasmid DNA, and the mixture was kept on ice for between 20 and 40 minutes. Cells were heat-shocked by placing tubes into a 42°C water bath for between 60 - 90 seconds, then placed back onto ice for a further 2 minutes. Cells were recovered in SOC broth and aerated by shaking for between 60 and 90 minutes at 37°C. SOC broth contained 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose. 150 µL of culture was spread onto nutrient agar plates containing the relevant antibiotic and incubated at 37°C overnight. Antibiotics used to maintain plasmids in transformants were 100 µg mL^{-1} ampicillin, 150 µg mL^{-1} erythromycin, 25 or 100 µg mL^{-1} kanamycin and 30 µg mL^{-1} chloramphenicol. To screen for transformants containing the pGEM®-T easy vector interrupted with a DNA insert, nutrient agar plates were supplemented with 80 µg mL^{-1} X-gal and 500 µM IPTG. The following day, transformants were restreaked for single colonies and incubated at 37°C overnight. To recover plasmids from transformed *E. coli* cells, transformants were inoculated into between 2 and 5 mL of LB containing the relevant antibiotics. Cultures were grown overnight with shaking at 37°C, then bacteria were pelleted by centrifugation at 12,000 to 16,000 rpm. Plasmid DNA was extracted from these cell pellets using the QIAprep® Spin Miniprep kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 50 µL elution buffer (supplied in kit) or double-distilled water and stored at -20°C.

2.16 Transformation of *Neisseria gonorrhoeae*: construction of single, double and triple mutants

Bacteria can acquire foreign DNA by three possible routes: a) conjugation; b) transduction; or c) transformation. For gonococci, conjugation is rare and there is no known phage that can successfully infect the bacterium, making transduction impossible (Seifert *et al.*, 1991). However, *N. gonorrhoeae* is one of the few species of bacteria that are naturally transformable, allowing the exchange of DNA between members of the *Neisseria* genus (Hamilton & Dillard, 2006). In order for transformation to occur, gonococci must be piliated and the DNA with which the gonococcus is to be transformed must contain a particular DNA uptake sequence (5′-GCCGTCTGAA-3′), which is inter-dispersed throughout the chromosome about 1900 times in members of the *Neisseria* genus (Biswas *et al.*, 1977; Davidsen *et al.*, 2004; Chung *et al.*, 2008). Plasmid DNA is often linearised after uptake by the gonococcus, making it difficult to maintain plasmids within the bacterium (Biswas *et al.*, 1986). Consequently, *in vitro* deletion and insertion of DNA sequences is achieved by homologous recombination onto the gonococcal chromosome using a high concentration of double-stranded linear DNA (Seifert *et al.*, 1991).

To construct single, double and triple mutants defective in *c*-type cytochromes and/or the lipid-associated azurin, Laz, a standard method was used. Bacteria were plated onto GCA plates containing 3 mM isoleucine to induce piliation, and grown for 24 h at 37°C (Larribe *et al.*, 1997). Piliated colonies were then restreaked onto isoleucine plates, and grown for an additional 24 h. Bacteria from these plates were swabbed into sterile universals containing 0.5 – 1 mL of GCB supplemented with 1% Kellogg's supplement and 10 mM $MgCl_2$, then 100

µL of this solution was dispensed by pipette onto the centre of a GCA plate. 50 to 100 µL of relevant DNA from a PCR reaction was added to the bacterial solution. Either plasmid or genomic DNA was used as a template for standard PCR reactions (Table 2.2) to create linear fragments of donor DNA, using primers specific to the region to be amplified. Plates were incubated upside-down at 37°C for 6 h, then bacteria were swabbed from these plates onto GCA plates containing the relevant antibiotic for selection. Antibiotics used to select for transformants were 2 µg mL^{-1} erythromycin, 100 µg mL^{-1} kanamycin and 1 µg mL^{-1} chloramphenicol. The recipient strains, template DNA and primers used to construct each single, double and triple mutant strain are shown in Table 2.5. The construction of DNA templates for transformation into gonococci are described in the following sections of Materials and Methods.

2.17 Construction of plasmids for ectopic complementation of *cycA* and *cycB* mutations

As described previously, plasmids are not stable in gonococci as they are often linearised on uptake (Biswas *et al.*, 1986). In order to complement the cytochrome c_4 and c_5 deletion mutants, ectopically expressed copies of the cytochrome c_4 and c_5 genes were therefore inserted into the chromosome under the control of a synthetic *lac* promoter rather than their native promoters. To allow the expression of the *cycA* and *cycB* gene products at unlinked loci on the chromosome of *N. gonorrhoeae* F62 strains, the Neisserial Insertional Complementation System (NICS) was used (Mehr & Seifert, 1998; Mehr *et al.*, 2000; Stohl *et al.*, 2005). This involved the construction of IPTG-inducible copies of *cycA* and *cycB* using the plasmid pGCC4 (kindly provided by C. Tang). To construct the IPTG-inducible copy of the *cycA*

Table 2.5 – *Neisseria gonorrhoeae* strains constructed in this work

Strain constructed	Recipient strain	Mutation added	DNA template*	Primers
AHGC100	F62	*kan*^{R}	pAH110	AHccoP_ds_F AHccoP_ds_R2a
AHGC101	F62	*ccoP* C368A *kan*^{R}	pAH111	AHccoP_ds_F AHccoP_ds_R2a
AHGC102	F62	*ccoP* C371A *kan*^{R}	pAH112	AHccoP_ds_F AHccoP_ds_R2a
AHGC103	F62	*ccoP* C371A H372A *kan*^{R}	pAH113	AHccoP_ds_F AHccoP_ds_R2a
AHGC104	F62	*ccoP* C368A C371A H372A *kan*^{R}	pAH114	AHccoP_ds_F AHccoP_ds_R2a
JCGC10	F62	*ΔaspC-lctP::placI-cycA*$^+$ *ermC*	pGCC4_cycA	pGCC4_F pGCC4_R
JCGC11	F62	*ΔaspC-lctP::placI-cycB*$^+$ *ermC*	pGCC4_cycB	pGCC4_F pGCC4_R
JCGC140	F62	*ΔaspC-lctP::placI-cccA*$^+$ 14 bp upstream, *ermC*	pGCC4_cccAb	pGCC4_F pGCC4_R
JCGC142	JCGC140 (*ΔaspC-lctP::placI-cccA*$^+$ 14 bp upstream *ermC*)	*cccA::cat*	pAH107	YLC552AUF YLC552CDR
JCGC150	F62	*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream, *ermC*	pGCC4_cccA	pGCC4_F pGCC4_R
JCGC151	F62	*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream *cccA* T-22A T-21A C-20G, *ermC*	pGCC4_cccA _loop	pGCC4_F pGCC4_R
JCGC152	JCGC150 (*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream, *ermC*)	*cccA::kan*^{R}	pYL13	YLC552AUF YLC552CDR
JCGC153	JCGC151 (*ΔaspC-lctP::placI-cccA*$^+$ 50 bp upstream *cccA* T-22A T-21A C-20G, *ermC*)	*cccA::kan*^{R}	pYL13	YLC552AUF YLC552CDR
JCGC216	JCGC861 (*cccA::cat*)	*cycP::ermC*	JCGC214 gDNA	ST_CycPFP3 ST_CycPRP1
JCGC310	F62	*cycB Δ*200-279*::ermC*	pAH131	AHC5-2int_F AHC5-2int_R
JCGC311	F62	*cycB Δ*200-279*::cat*	pAH132	AHC5-2int_F AHC5-2int_R
JCGC315	JCGC310 (*cycB Δ*200-279*::ermC*)	*cccA::cat*	pAH107	YLC552AUF YLC552CDR
JCGC317	JCGC310 (*cycB Δ*200-279*::ermC*)	*laz::cat*	pAH140	NTAZUFOR NTAZUREV
JCGC320	AHGC101 (*ccoP* C368A *kan*^{R})	*cycB Δ*200-279*::ermC*	pAH131	AHC5-2int_F AHC5-2int_R
JCGC321	AHGC101 (*ccoP* C368A *kan*^{R})	*cycB Δ* 200-279*::cat*	pAH132	AHC5-2int_F AHC5-2int_R
JCGC330	AHGC101 (*ccoP* C368A *kan*^{R})	*cccA::cat*	pAH107	YLC552AUF YLC552CDR
JCGC335	AHGC101 (*ccoP* C368A *kan*^{R})	*laz::cat*	pAH140	NTAZUFOR NTAZUREV
JCGC340	AHGC101 (ccoP C368A kan^R)	*cycA::ermC*	pNT102	NTCYC4 FOR NTCYC4 REV
JCGC350	JCGC320 (*ccoP* C368A *kan*^{R} *cycB Δ* 200-279*::ermC*)	*cccA::cat*	pAH107	YLC552AUF YLC552CDR
JCGC351	JCGC340 (*ccoP* C368A *kan*^{R}	*cccA::cat*	pAH107	YLC552AUF

Strain constructed	Recipient strain	Mutation added	DNA template*	Primers
	cycA::ermC)			YLC552CDR
JCGC352	JCGC320 (*ccoP* C368A *kan*^{R} *cycB Δ*200-279*::ermC*)	*laz::cat*	pAH140	NTAZUFOR NTAZUREV
JCGC355	JCGC360 (*ccoP* C368A *kan*^{R} *cycP::ermC*)	*cycB Δ*200-279*::cat*	pAH132	AHC5-2int_F AHC5-2int_R
JCGC807	F62	*cccA::*3xFLAG *kan*^{R}	pAHC2F	AHC2AUFEcoRI AHC2CDRNheI
JCGC820	JCGC10 (*ΔaspC-lctP::placI-cycA*$^+$ *ermC*)	*cycA::kan*^{R}	JCGC801 gDNA	NTCYC4 FOR NTCYC4 REV
JCGC860	F62	*cycB::cat*	pAH105	YLC5AUF YLC5CDR
JCGC861	F62	*cccA::cat*	pAH107	YLC552AUF YLC552CDR
JCGC870	JCGC11 (*ΔaspC-lctP::placI-cycB*$^+$ *ermC*)	*cycB::cat*	pAH105	YLC5AUF YLC5CDR
JCGC880	JCGC870 (*cycB::cat ΔaspC-lctP::placI-cycB*$^+$ *ermC*)	*cycA::kan*^{R}	JCGC801 gDNA	NTCYC4 FOR NTCYC4 REV
JCGC893	F62	*laz::cat*	pAH140	NTAZUFOR NTAZUREV

*gDNA refers to *N. gonorrhoeae* F62 genomic DNA.

gene, the gonococcal *cycA* coding region and 39 bp of upstream DNA and 103 bp of downstream DNA were amplified by PCR from *N*. *gonorrhoeae* strain F62 genomic DNA using the primers NC_C4F and NC_C4R, respectively, which were flanked by *PacI* restriction sites. The plasmid pGCC4_cycA was constructed by digesting the pGCC4 plasmid and the aforementioned *cycA* insert with *PacI*, then ligating the insert into the plasmid. The orientation of the insert was verified using primer AHpGCC4PacIns.

To construct the IPTG-inducible copy of the *cycB* gene, the gonococcal *cycB* coding region and 28 bp of upstream DNA and 149 bp of downstream DNA were amplified by PCR from *N*. *gonorrhoeae* strain F62 genomic DNA using the primers AHCptC5F and AHCptC5R, which were flanked by *PacI* and *FseI* restriction sites, respectively. The plasmid pGCC4_cycB was constructed by digesting the pGCC4 plasmid and the aforementioned *cycB* insert with both *PacI* and *FseI*, then ligating the insert into the plasmid. The orientation of the insert was verified using primer AHpGCC4PacIns.

In order to transfer the IPTG-inducible copies of *cycA* and *cycB* into the gonococcus, linear fragments were required. These included regions of homology to the chromosome at alternative loci to the original copies of the *cycA* and *cycB* genes, as well as an erythromycin resistance cassette for selection of transformants. These linear fragments were created by PCR amplification of the required region using primers pGCC4_F and pGCC4_R. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for erythromycin resistance. Candidates for mutants containing the IPTG-inducible *cycA* or *cycB* cassettes were screened by PCR and sequenced

using the primer AHpGCC4PacIns.

2.18 Construction of a plasmid for overexpression of cytochrome c_2

To construct the IPTG-inducible copy of the *cccA* gene, the gonococcal *cccA* coding region and 14 bp of upstream DNA and 204 bp of downstream DNA were amplified by PCR from *N. gonorrhoeae* strain F62 genomic DNA using the primers AHCptC2Fb and AHCptC2R, which were flanked by *PacI* and *FseI* restriction sites, respectively. The plasmid pGCC4_cccAb was constructed by digesting the pGCC4 plasmid and the aforementioned *cccA* insert with *PacI* and *FseI*, then ligating the insert into the plasmid. The orientation of the insert was verified using primer pGCC4PacIns. In order to transfer the IPTG-inducible copy of *cccA* into the gonococcus, linear fragments were required. These included regions of homology to the chromosome at an alternative locus to the original copy of the *cccA* gene, as well as an erythromycin resistance cassette for selection of transformants. These linear fragments were created by PCR amplification of the required region using primers pGCC4_F and pGCC4_R and the plasmid pGCC4_cccAb as a template. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for erythromycin resistance. Candidates for mutants containing the IPTG-inducible *cccA* cassette were screened by PCR and sequenced using the primer AHpGCC4PacIns.

2.19 Site-directed mutagenesis of the potential stem loop structure upstream of the *cccA* coding sequence

To construct the IPTG-inducible copy of the *cccA* gene containing the upstream potential

stem loop structure, the gonococcal *cccA* coding region and 50 bp of upstream DNA and 204 bp of downstream DNA were amplified by PCR from *N*. *gonorrhoeae* strain F62 chromosomal DNA using the primers AhCptC2F and AHCptC2R, which were flanked by *PacI* and *FseI* restriction sites, respectively. The plasmid pGCC4_cccA was constructed by digesting the pGCC4 plasmid and the aforementioned *cccA* insert with *PacI* and *FseI*, then ligating the insert into the plasmid. The orientation of the insert was verified using primer pGCC4PacIns. In order to transfer the IPTG-inducible copy of *cccA* into the gonococcus, linear fragments were required. These included regions of homology to the chromosome at an alternative locus to the original copy of the *cccA* gene, as well as an erythromycin resistance cassette for selection of transformants. These linear fragments were created by PCR amplification of the required region using primers pGCC4_F and pGCC4_R and the plasmid pGCC4_cccA as a template. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for erythromycin resistance. Candidates for mutants containing the IPTG-inducible *cccA* cassette were screened by PCR and sequenced using the primer AHpGCC4PacIns.

To mutate the potential stem loop structure located upstream of the *cccA* gene, the Stratagene QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used. The primers AHC2loopSDM_Fb and AHC2loopSDM_Rb were designed to change the bases T-22 to A, T-21 to A and C-20 to G (where the - sign denotes the number of base pairs upstream of the ATG start codon of the *cccA* coding sequence). This would prevent this region of the mRNA molecule from pairing with part of the Shine-Dalgarno site. The primer pair AHC2loopSDM_Fb and AHC2loopSDM_Rb were used to mutagenise the plasmid

pGCC4_cccA to create the plasmid pGCC4_cccA_loop. Linear DNA fragments were amplified from pGCC4_cccA_loop using primers AHpGCC4_F and AHpGCC4_R. These DNA fragments included regions of homology to the chromosome at an alternative locus to the original copy of the *cccA* gene, as well as an erythromycin resistance cassette for selection of transformants. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for erythromycin resistance. Erythromycin resistant colonies were screened by PCR for the presence of the chromosomal insert, and sequenced using the primer AHpGCC4PacIns to verify successful recombination.

2.20 Construction of a gonococcal cytochrome c_2 deletion mutation conferring chloramphenicol resistance

The *cat* gene (conferring chloramphenicol resistance) was amplified from pLES940 using primers AHCatAgeIF and AHCatAgeIR, which introduced *AgeI* sites at each end of the fragment. Both the PCR fragment containing the *cat* gene and pYL12 were digested with *AgeI* and ligated to form pAH107. Linear DNA containing the *cat* gene flanked by DNA upstream and downstream of the *cccA* coding region was produced by PCR using primers YLC552AUF and YLC552CDR. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for chloramphenicol resistance. Candidate clones were confirmed by sequencing using the primer YLC552AUF.

2.21 Construction of a gonococcal cytochrome c_5 deletion mutation conferring chloramphenicol resistance

The *cat* gene (conferring chloramphenicol resistance) was amplified from pLES940 using primers AHCatAgeIF and AHCatAgeIR, which introduced *AgeI* sites at each end of the fragment. Both the PCR fragment containing the *cat* gene and pYL6 were digested with *AgeI* and ligated to form pAH105. Relevant *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with linear DNA produced by PCR using primers YLC5AUF and YLC5CDR and screened for chloramphenicol resistance. Mutants were confirmed by sequencing using primer YLC5AUF.

2.22 Construction of a gonococcal *laz* deletion mutation conferring chloramphenicol resistance

The *cat* gene (conferring chloramphenicol resistance) was amplified from pLES940 using primers AHCatAgeIF and AHCatAgeIR, which introduced *AgeI* sites at each end of the fragment. Plasmid pNT502 was digested with *AgeI* to release the kan^R gene. The PCR fragment containing the *cat* gene was digested with *AgeI*, then ligated into the pNT502 vector lacking the kan^R gene, forming pAH140. Relevant *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with linear DNA produced by PCR using primers NTAZUFOR and NTAZUREV to create strain JCGC315. Candidates were screened for chloramphenicol resistance and confirmed by sequencing using the primer NTAZUFOR.

2.23 Site-directed mutagenesis of the third heme-binding domain of CcoP

To obtain mutants defective in the third heme-binding motif of CcoP, the gonococcal ccoP-coding region and 415 bp of upstream DNA and 219 bp of downstream DNA were amplified by PCR from *N. gonorrhoeae* strain F62 genomic DNA using the primers AHccoP_ds_F and AHccoP_ds_R2a. The plasmid pAH100 was constructed by ligating the PCR fragment into pGEM T-Easy vector. The third heme-binding motif of CcoP was mutated to form CcoP C368A, CcoP C371A, CcoP C371A H372A and CcoP C368A C371A H372A using the Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene) and primer pairs AHccoP_C368A_F/R, AHccoP_C371A_F/R, AHccoP_C371A-H372A_F/R and AHccoP_C368A-C371A-H372A_F/R, respectively. This created the plasmids pAH100, pAH101, pAH102, pAH103 and pAH104, respectively. Two natural *BspEI* restriction sites were located 237 and 254 bp downstream from codons for the third heme-binding motif, and 36 bp downstream of the stop codon of the *ccoP* gene. The *kan*^{R} gene was amplified from pYL15 using primers AHKanBspEI-F and AHKanBspEI-Rb, which introduced *BspEI* sites at each end. The *kan*^{R} gene was ligated into the *BspEI* sites of pAH100, pAH101, pAH102, pAH103 and pAH104 to create the constructs pAH110, pAH111, pAH112, pAH113 and pAH114, respectively. Linear DNA fragments were amplified from pAH110, pAH111, pAH112, pAH113 or pAH114 using primers AHccoP_ds_F and AHccoP_ds_R2a. The DNA fragments contained the *kan*^{R} gene flanked by DNA homologous to the coding region of the *ccoP* gene and non-coding DNA upstream and downstream of the gene in addition to the gonococcal uptake sequence. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for kanamycin resistance. Kanamycin-resistant colonies were screened by PCR for the presence of the kanamycin resistance cassette, and the PCR product

was then sequenced using the primer AHccoPKan_check to verify the recombination of the specific site-directed mutation.

2.24 Construction of mutants defective in the second domain of cytochrome c_5

Crossover PCR was used to construct a truncated version of cytochrome c_5. Two fragments of DNA were amplified from *N*. *gonorrhoeae* strain F62 chromosomal DNA. Primers AHC5-2ndAUF and AHC5-2ndBURb were used to amplify a region of DNA including 502 bp of non-coding DNA upstream of the *cycB* gene and 595 bp of coding DNA of the *cycB* gene. Primer AHC5-2ndBURb incorporated a stop codon, to allow the translation of the truncated version of the cytochrome c_5 protein. Primers AHC5-2ndDDF and AHC5-2ndCDR were used to amplify 612 bp of non-coding DNA downstream of the *cycB* gene. The fragments contained regions of sequence identity at the 3' end of the upstream fragment and at the 5' end of the downstream fragment that included an *AgeI* restriction site. The fragments were able to anneal to one another during a subsequent PCR reaction using primers AHC5-2ndAUF and AHC5-2ndCDR to form a larger fragment. This contained the upstream non-coding and coding region immediately followed by the downstream non-coding region with an *AgeI* restriction site located at the cross-over point. To create a larger amount of this specific fragment, the fragment was gel-extracted and the DNA amplified by PCR using primers AHC5-2int_F and AHC5-2int_R. The plasmid pAH130 was constructed by ligating this enlarged PCR fragment into pGEM® T-Easy vector. The *ermC* gene was amplified from JCGC100 using the primers NTERY FOR and NTERY REV, which introduced *AgeI* sites at each end. The *cat* gene was amplified from pLES940 using the primers AHCatAgeIF and AHCatAgeIR, which introduced *AgeI* sites at each end. The *ermC* gene fragment, *cat* gene

fragment and pAH130 were digested with *AgeI*, then either the *ermC* gene or the *cat* gene was ligated into the *AgeI* site of pAH130 to create the constructs pAH131 and pAH131C, respectively. Linear DNA fragments were amplified from pAH131 and pAH131C using primers AHC5-2int_F and AHC5-2int_R. These DNA fragments contained either the *ermC* gene or *cat* gene flanked by DNA homologous with the non-coding and coding regions of DNA upstream and the non-coding DNA downstream of the *cycB* gene in addition to the gonococcal uptake sequence. Relevant piliated *Neisseria gonorrhoeae* F62 strains (see Table 2.5) were transformed with these linear fragments and screened for either erythromycin or chloramphenicol resistance. After 3 days of growth on GC agar plates supplemented with erythromycin or chloramphenicol, purified colonies were screened by PCR for the truncation of the *cycB* gene. Candidate clones of JCGC310 or JCGC311 were confirmed by sequencing using the primer AHC5-2int_F.

2.25 Construction of 3xFLAG-tagged cytochrome c_2

To create a FLAG-tagged version of cytochrome c_2, linear fragments of DNA containing 336bp of DNA located downstream of the *cccA* coding region were amplified from *N. gonorrhoeae* strain F62 genomic DNA by PCR using primers AHC2CDRNheI and AHC2DDFXhoI. The resulting fragments and the pDOC-F plasmid were digested with *NheI* and *XhoI* enzymes, then ligated to form pAHC2FCD. Primers AHC2AUFEcoRI and AHC2BURKpnI were used to amplify by PCR 435bp of upstream and 465bp of coding DNA of the *cccA* gene from F62 genomic DNA. The resulting fragments and the pAHC2FCD plasmid were digested with *EcoRI* and *KpnI* enzymes, then the products were ligated to form pAHC2F. Relevant piliated *N. gonorrhoeae* F62 strains (see Table 2.5) were transformed with

a linear DNA fragment amplified by PCR from pAHC2F using primers AHC2AUFEcoRI and AHC2CDRNheI. This DNA fragment contained the *cccA* upstream DNA and coding sequence, followed by an in-frame 3xFLAG tag, the *kan*^{R} gene then DNA downstream of the *cccA*. This construct would therefore allow homologous recombination of the FLAG-tagged version of cytochrome c_2 onto the F62 chromosome. After 3 days of growth on GC agar plates supplemented with kanamycin, purified colonies were screened by PCR for the FLAG-tagged version of the *cccA* gene. Candidate clones were confirmed by sequencing using the primer AHC2DDFXhoI.

2.26 DNA sequencing

Fully automated DNA sequencing was provided by the Functional Genomics Laboratory (University of Birmingham). DNA to be sequenced was quantified and the optimum concentration recommended by the Functional Genomics Laboratory was used. Relevant primers were added at a concentration of 3.2 pmol, and the total volume was made to 10 µL using sterile double-distilled water. The genomics facility then added Terminator Reaction Mix to a total volume of 20 µL, and the samples were run on an ABI 3730 DNA analyser. The Chromas program (Technelysium Pty Ltd) was used to analyse the DNA sequences produced.

2.27 Reproducibility of data

Growth data were taken from a minimum of two biological replicates grown on at least two different days, and were typically grown in duplicate. The number of replicates of each growth experiment is indicated in the figure legends. For oxygen reduction assays, at least

five measurements were taken per strain from at least two biologically replicated cultures grown on different days. Typically, three measurements were taken from each culture on each day. For nitrite reduction assays, at least four measurements were taken per strain from at least two biologically replicated cultures grown on different days. Typically, two measurements were taken from each culture on each day. In many cases, far more experiments were replicated, e.g. the F62 parent strain and other control strains. The number of replicates for each biochemical assay is indicated in figure legends.

2.28 Statistical analysis

Mean values, standard deviation and standard error of the mean were calculated using the Microsoft Excel program. The Student's *t*-test (Welch's adaptation) was used to determine whether the differences in the means of two different sets of data were statistically significant. The R program (www.r-project.org) was used to calculate Student's *t*-test *P*-values. If a *P*-value of less than 0.05 was found between two sets of data, it was considered that the difference between the mean values of these data was significant. If the *P*-value of two sets of data was greater than 0.05, it was interpreted that there was no significant difference between the mean values of the two data sets, i.e. the mean values can be considered to be comparable.

2.29 Computational analysis

The published DNA sequence of *Neisseria gonorrhoeae* strain FA1090 (GenBank accession number AE004969) was acquired from the Gonococcal Genome Sequencing Project

supported by USPHS/NIH grant #AI38399, and B.A. Roe, L. Song, S. P. Lin, X. Yuan, S. Clifton, Tom Ducey, Lisa Lewis and D.W. Dyer at the University of Oklahoma (found at URL http://stdgen.nothwestern.edu). Other bacterial DNA and amino acid sequences were acquired using SRS@EBI (European Bioinformatics Institute) (Harte *et al.*, 2004). DNA and amino acid sequence alignments were made using EMBOSS Needle, EMBOSS Water or Clustal W2 programs (all European Bioinformatics Institute) (Harte *et al.*, 2004). The BLAST program (BLAST@NCBI) was used to search for DNA or amino acid sequences with similarity to a query sequence (Altschul *et al.*, 1990). The WatCut program (URL http://watcut.uwaterloo.ca/watcut/watcut/template.php) was used to locate restriction sites within a query DNA sequence. Published protein structures were acquired from the RCSB Protein Data Bank (URL www.pdb.org), and the PDB viewer program (Guex & Peitsch, 1997) was used to generate models of these protein structures.

Chapter 3

Electron transfer to oxygen

3.1 Introduction

The gonococcus is a prolific source of multiple *c*-type cytochromes compared to other species of bacteria and to heart mitochondria (Winter & Morse, 1975; Whitehead *et al.*, 2007). Some of these gonococcal cytochromes have no known functions. The publication of the complete genome sequences of pathogenic and non-pathogenic *Neisseria* revealed that there is only one cytochrome oxidase that is closely related to the cytochrome cbb_3 oxidase of *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, and *Azotobacter vinelandii* (Preisig *et al.*, 1993; Thöny-Meyer *et al.*, 1994; Rey & Maier, 1997). Little is known about how the electron transfer chains of the gonococcus are organized. Cytochrome cbb_3 oxidase from *Bradyrhizobium japonicum* has a very low K_m for oxygen (K_m = 7 nM), which is essential to reduce the oxygen concentration in root nodules to the nanomolar range, thus protecting nitrogenase from inactivation by oxygen (Preisig *et al.*, 1993; Preisig *et al.*, 1996). In both *B. japonicum* and the *Neisseriaceae*, it is encoded by the *ccoNOPQ* operon (originally designated the *fixNOPQ* operon, for nitrogen fixation), in which the *ccoO* and *ccoP* genes encode *c*-type cytochromes. Genes for six other gonococcal *c*-type cytochromes include *cycP*, encoding cytochrome *c*', which was implicated in protection against NO toxicity (Turner *et al.*, 2005); *ccp*, encoding a cytochrome *c* peroxidase (Turner *et al.*, 2003); *petC*, encoding the cytochrome c_1 component of the cytochrome bc_1 complex; and three genes, *cccA*, *cycA*, and *cycB*, encoding cytochromes of unknown function that have been designated cytochromes c_2, c_4, and c_5, respectively. By staining proteins separated by SDS-PAGE for covalently bound heme, it has been shown that six *c*- type cytochromes accumulate during aerobic growth, including cytochromes c_4 and c_5 (Strange & Judd, 1994; Turner *et al.*, 2003; Turner *et al.*, 2005). A seventh *c*-type cytochrome, Ccp, is expressed at low levels during

oxygen-limited growth (Lissenden *et al.*, 2000; Turner *et al.*, 2003; Whitehead *et al.*, 2007). However, cytochrome c_2 has not been visualized either in gonococci or in the closely related pathogen, *Neisseria meningitidis* (Deeudom *et al.*, 2006); consequently, its role in gonococcal electron transfer pathways remains to be defined. Cytochromes c_4 and c_5 were first identified in the nitrogen-fixing bacterium *Azotobacter vinelandii*, where they were proposed to provide parallel pathways for electron transfer between the cytochrome bc_1 complex and cytochrome oxidase (Haddock & Jones, 1977). Many years later, it was tentatively suggested that they provide two independent pathways to a cytochrome cbb_3-type oxidase (Rey & Maier, 1997). This offers a possible explanation for the conflicting previous proposals concerning the organization of the gonococcal respiratory chain, namely, that there are branched electron transfer pathways to oxygen that diverge at the cytochrome bc_1 complex but terminate with the same cytochrome oxidase, cbb_3. The current study was therefore designed to determine whether gonococcal cytochromes c_4 and c_5 provide alternative pathways for electron transfer between cytochrome bc_1 and cytochrome cbb_3 oxidase and whether cytochrome c_2 also provides an alternative electron transfer pathway to cytochrome oxidase. The results obtained provide fascinating insights into why a pathogen with a microaerobic lifestyle maintains such a high respiratory capacity compared to other Gram negative bacteria and is such a prolific source of electron transfer components (Jurtshuk & Milligan, 1974; Winter & Morse, 1975).

The work in this chapter is based on a paper by Li *et al.*, 2010, of which the author of this work is joint first author. Experiments in Li *et al.*, 2010 were completed in collaboration with co-authors Dr Ying Li, Dr Derrick Squire and Dr Nick Tovell. Additional data not found in the

aforementioned paper results from later research by the author of this current work, who completed all of the experiments reported in the results section of this chapter. Additional experiments not completed by the author are discussed at the end of the chapter.

3.2 Rates of oxygen reduction and myxothiazol inhibition by single mutants defective in cytochromes c_2, c_4, and c_5

Lactate is the preferred physiological electron donor for gonococcal respiration, with rates of oxygen reduction by the parental strain F62 being approximately three times greater in the presence of lactate than in the presence of glucose (Morse *et al.*, 1979; Whitehead *et al.*, 2007). The rate of lactate-dependent oxygen reduction by the *cycA* mutant that lacks cytochrome c_4 was 16% lower than that of the parent, and the corresponding rate for the *cycB* mutant that lacks cytochrome c_5 was 20% lower than that of the parent (Table 3.1). In contrast, the rate of oxygen reduction by the *cccA* mutant that lacks cytochrome c_2 was almost 26% greater than that of the parent. These data were consistent with the proposal that cytochromes c_4 and c_5, but not cytochrome c_2, are involved in electron transfer to the terminal cytochrome cbb_3 oxidase.

The artificial electron donor TMPD donates electrons to the cytochrome c_1 component of the cytochrome bc_1 complex, saturating the terminal components of the respiratory chain with reducing equivalents (Winter & Morse, 1975). Mutations that result in the loss of the capacity for electron transfer between cytochrome bc_1 and cytochrome oxidase should therefore also be reflected in lower rates of TMPD-dependent oxygen reduction. Furthermore, the deletion of the gene for one of two components of the electron transfer

A

Strain	Lactate rate	No. replicates	% F62 lactate rate	Lactate + myxothiazol rate	No. replicates	% inhibition by myxothiazol
F62	209 ± 4	19	100	152 ± 6	11	27
Δc_4	176 ± 8	15	84	89 ± 6	9	49
Δc_5	167 ± 4	7	80	65 ± 7	5	61
Δc_2	264 ± 17	12	126	199 ± 12	5	25
Δc_2 Δc_4	224 ± 10	14	107	112 ± 9	10	50
Δc_2 Δc_5	118 ± 5	5	57	51 ± 4	5	57

Strain	Ascorbate + TMPD rate	No. replicates	% WT ascorbate + TMPD rate
F62	1351 ± 30	8	100
Δc_4	1030 ± 98	10	76
Δc_5	915 ± 72	6	68
Δc_2	1459 ± 40	6	108
Δc_2 Δc_4	1144 ± 42	10	85
Δc_2 Δc_5	696 ± 30	5	52

B

Strain	*P*-value
F62	
Δc_4	0.0014 (F62)
Δc_5	9.66×10^{-7} (F62)
Δc_2	0.0071 (F62)
Δc_2 Δc_4	0.1596 (F62), 0.0007 (Δc_4), 0.0513 (Δc_2)
Δc_2 Δc_5	5.92×10^{-5} (F62), 4.09×10^{-5} (Δc_5), 1.55×10^{-6} (Δc_2)

Table 3.1 – Rates of oxygen reduction by washed bacterial suspensions of strains F62, JCGC800 (Δc_4), JCGC850 (Δc_5), JCGC851 (Δc_2), JCGC853 (Δc_2 Δc_4) and JCGC852 (Δc_2 Δc_5)

A) Rates of oxygen reduction by cultures grown under oxygen-sufficient conditions. Cultures were grown under oxygen-sufficient conditions until the optical density at 650 nm reached a value between 0.8-1. Bacteria were then harvested, washed and their ability to reduce oxygen was measured using a Clark-type oxygen electrode. Either lactate or ascorbate + TMPD were used as the electron donor, and, where relevant, 1 µM myxothiazol was added. Rates are nmol of oxygen reduced min^{-1}. (mg of bacterial biomass) $^{-1}$ ± the standard error of the mean. The number in brackets indicates the number of replicates, which were measured from at least two biologically replicated cultures.

B) *P*-values (calculated using the Welch's Student's *t*-test) comparing rates of oxygen reduction by strains using lactate as an electron donor. *P*-values are relative to the strain shown in brackets.

pathway would allow the capacity of the remaining component to be estimated. Oxygen reduction by ascorbate-TMPD was much more rapid than that by lactate for all four strains (Table 3.1), but rates for the cytochrome c_4 and c_5 mutants were again 24% and 32% lower, respectively, than that of the parent. This difference provided the first indication that the maximum rate of electron transfer via cytochrome c_5 might be greater than that through cytochrome c_4. The rate of ascorbate-TMPD oxidation by the *cccA* mutant was again slightly higher than that of the parent (Table 3.1).

Myxothiazol specifically inhibits electron transfer between ubiquinol and the cytochrome bc_1 complex by binding competitively to the quinol-binding site of the cytochrome bc_1 complex (Von Jagow *et al.*, 1984; Pember *et al.*, 2005). Deeudom *et al.* previously showed that *Neisseria meningitidis* respiration is completely inhibited by 1 µM myxothiazol (Deeudom *et al.*, 2006). The rates of oxygen reduction by the gonococcal cytochrome c_4 and c_5 mutants were strongly inhibited by 1 µM myxothiazol, with the *cycB* mutant defective in cytochrome c_5 being slightly more sensitive than the *cycA* mutant defective in cytochrome c_4 (Table 3.1). Lactate oxidation by the *cccA* mutant was also sensitive to myxothiazol, but less sensitive than the parental strain or either mutant. Even 20 µM myxothiazol still only partially inhibited gonococcal respiration. Although 1 µM myxothiazol was sufficient to inhibit meningococcal electron transfer to oxygen completely (Deeudom *et al.*, 2006), an isolated membrane fraction rather than intact bacteria was used for the meningococcal experiments, so the lack of complete inhibition in the current study is not surprising.

3.3 Complementation of the *cycA* and *cycB* mutations by ectopic expression of IPTG-inducible *cycA* and *cycB* genes

In order to complement the cytochrome c_4 and c_5 deletion mutants, ectopically expressed copies of the cytochrome c_4 and c_5 genes were inserted into the chromosome under the control of a synthetic *lac* promoter rather than their native promoters. To allow the expression of the *cycA* and *cycB* gene products at unlinked loci on the chromosome of *N. gonorrhoeae* F62 strains, the Neisserial Insertional Complementation System (NICS) was used (Mehr & Seifert, 1998; Mehr *et al.*, 2000; Stohl *et al.*, 2005). IPTG-inducible copies of either *cycA* or *cycB* were integrated between the *lctP* and *aspC* genes on the chromosomes of parental strain F62 and the *cycA* or *cycB* mutant, respectively. The insertion of the NICS complementation system onto the gonococcal chromosome confers erythromycin resistance to resulting transformants. As the cytochrome c_5 deletion mutant also conferred resistance to erythromycin, an alternative antibiotic resistant cytochrome c_5 mutant was required. This mutant was constructed by replacing the *cycB* coding sequence with a chloramphenicol resistance cassette (*cat*). The *c*-type cytochrome complement of the resulting mutant was then checked to confirm that the c_5*::cat* mutant was unable to synthesise cytochrome c_5. Cultures of strain F62 and JCGC860 (c_5*::cat*) were grown under oxygen-limited conditions and samples of these cultures were taken after 6 hours. The resulting bacterial pellets were separated by SDS-PAGE, and the gel was stained for the presence of covalently bound heme. In strain JCGC860, the band corresponding to cytochrome c_5 was absent, confirming that the *cycB* gene had been deleted (Figure 3.1).

Strains JCGC10 (c_4I, i.e. containing the IPTG-inducible copy of cytochrome c_4 at the

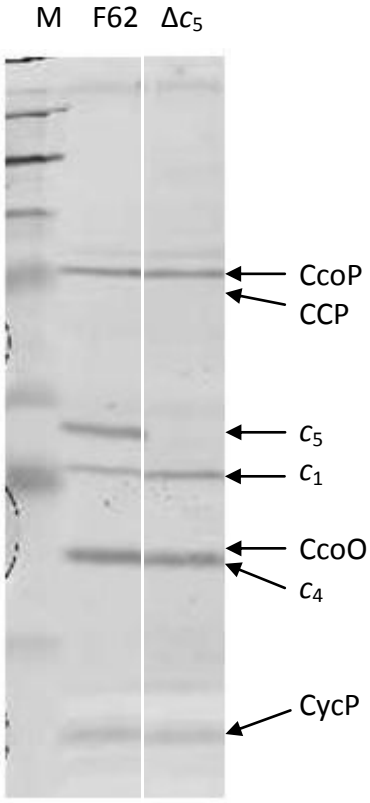


Figure 3.1 – SDS-PAGE gel stained for the presence of covalently bound heme in strains F62 and JCGC860 (Δc_5::*cat*)

Cultures were grown under oxygen-sufficient conditions and samples were taken after 6 hours of growth.

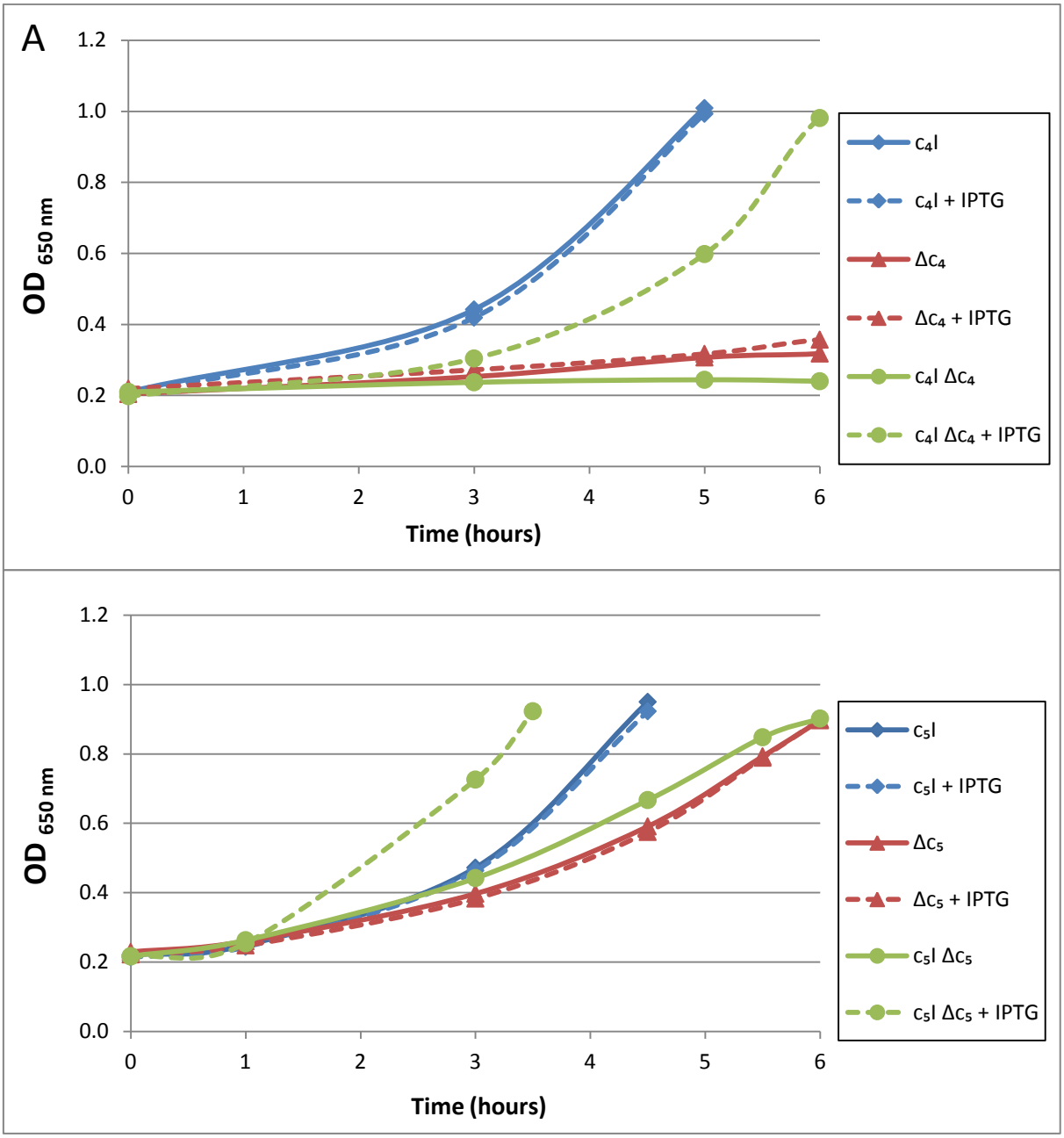
alternative chromosomal locus), JCGC801 (Δc_4) and JCGC820 (c_4I Δc_4) were grown under oxygen-sufficient conditions in the presence or absence of IPTG. IPTG had no effect on the growth of *cycA*$^+$ strain JCGC10, but the effect of the restoration of the *cycA*$^+$ phenotype on the growth of complemented *cycA* mutant strain JCGC820 was dependent upon the addition of IPTG (Figure 3.2A). The oxygen sensitivity of the original *cycA* mutant was unaffected by the addition of IPTG (Figure 3.2A). Similar results were obtained with the corresponding *cycB* derivatives. IPTG stimulated the oxygen-sufficient growth of complemented *cycB* strain JCGC870 but had no effect on the growth of the original *cycB* mutant strain JCGC860 or *cycB*$^+$ strain JCGC11 (Figure 3.2B). Proteins from samples harvested at the late exponential phase of growth of each of these cultures were separated by SDS-PAGE and stained for covalently bound heme (Figure 3.3). Ectopically expressed copies of *cycA*$^+$ or *cycB*$^+$ enhanced the staining intensity of the cytochrome c_4 or cytochrome c_5 band of the *cycA*$^+$ or *cycB*$^+$ strain and restored *cycA*$^+$ or *cycB*$^+$ synthesis to the corresponding mutant strains (Figure 3.3A and 3.3B). Furthermore, rates of oxygen reduction by the complemented c_4 and c_5 strains were identical to those of the parental strain following growth in the presence, but not in the absence, of IPTG (Figure 3.4A and 3.4B).

3.4 Phenotypes of double mutants defective in cytochromes c_2 and c_4 or cytochromes c_2 and c_5

The results presented above implicated both cytochromes c_4 and c_5 in electron transfer between the cytochrome bc_1 complex and cytochrome cbb_3 oxidase but suggested that cytochrome c_2 either is not involved in aerobic respiration or, at most, plays only a very minor role, at least under the growth conditions used in these experiments. The cytochrome

Figure 3.2 – Oxygen-sufficient growth of complemented cytochrome c_4 and c_5 deletion mutant strains

A) Oxygen-sufficient growth of strains JCGC10 (labelled c_4I), JCGC801 (labelled Δc_4) and JCGC820 (labelled c_4I Δc_4) in the absence and presence of IPTG. Continuous lines indicate that no IPTG was added to cultures, whereas dotted lines indicate cultures where 1 mM IPTG was added immediately post-inoculation. Growth curves shown are representative of duplicate cultures from two independent growth experiments.
B) Oxygen-sufficient growth of strains JCGC11 (labelled c_5I), JCGC860 (labelled Δc_5) and JCGC870 (labelled c_5I Δc_5) in the absence and presence of IPTG, indicated as above. Growth curves shown are representative of duplicate cultures from two independent growth experiments.


A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 650 nm
0
1
2
3
4
5
6
Time (hours)
c4I
c4I + IPTG
Δc4
Δc4 + IPTG
c4I Δc4
c4I Δc4 + IPTG
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 650 nm
0
1
2
3
4
5
6
Time (hours)
c5I
c5I + IPTG
Δc5
Δc5 + IPTG
c5I Δc5
c5I Δc5 + IPTG

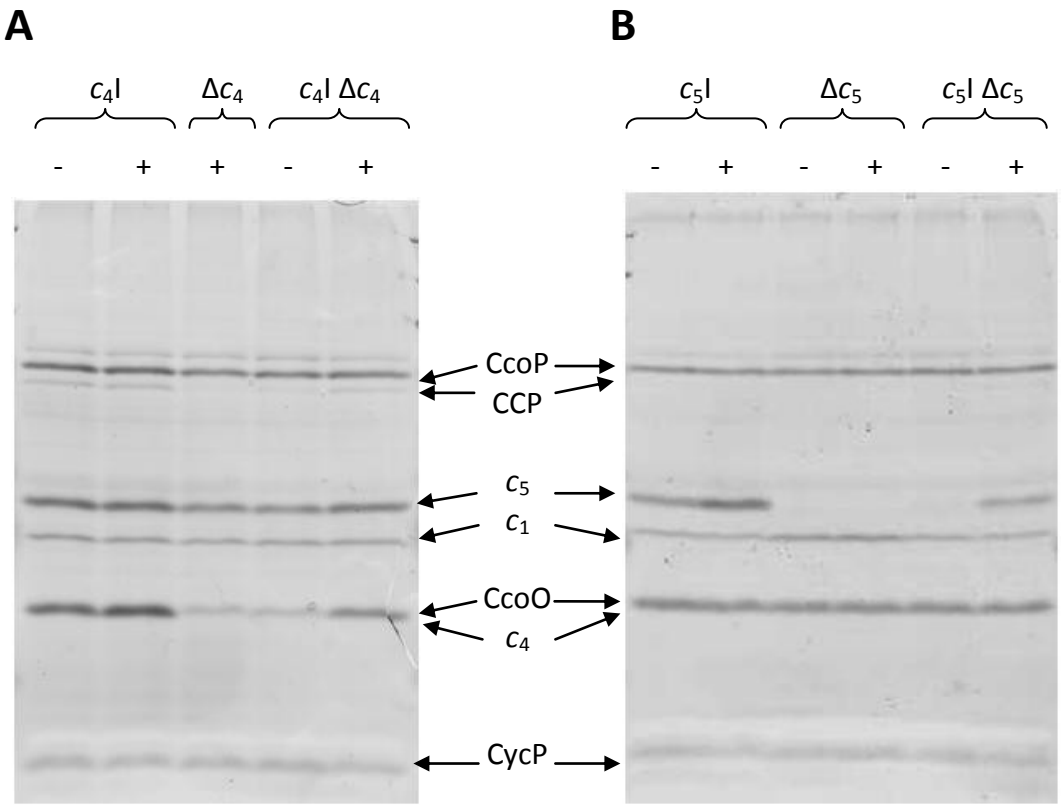


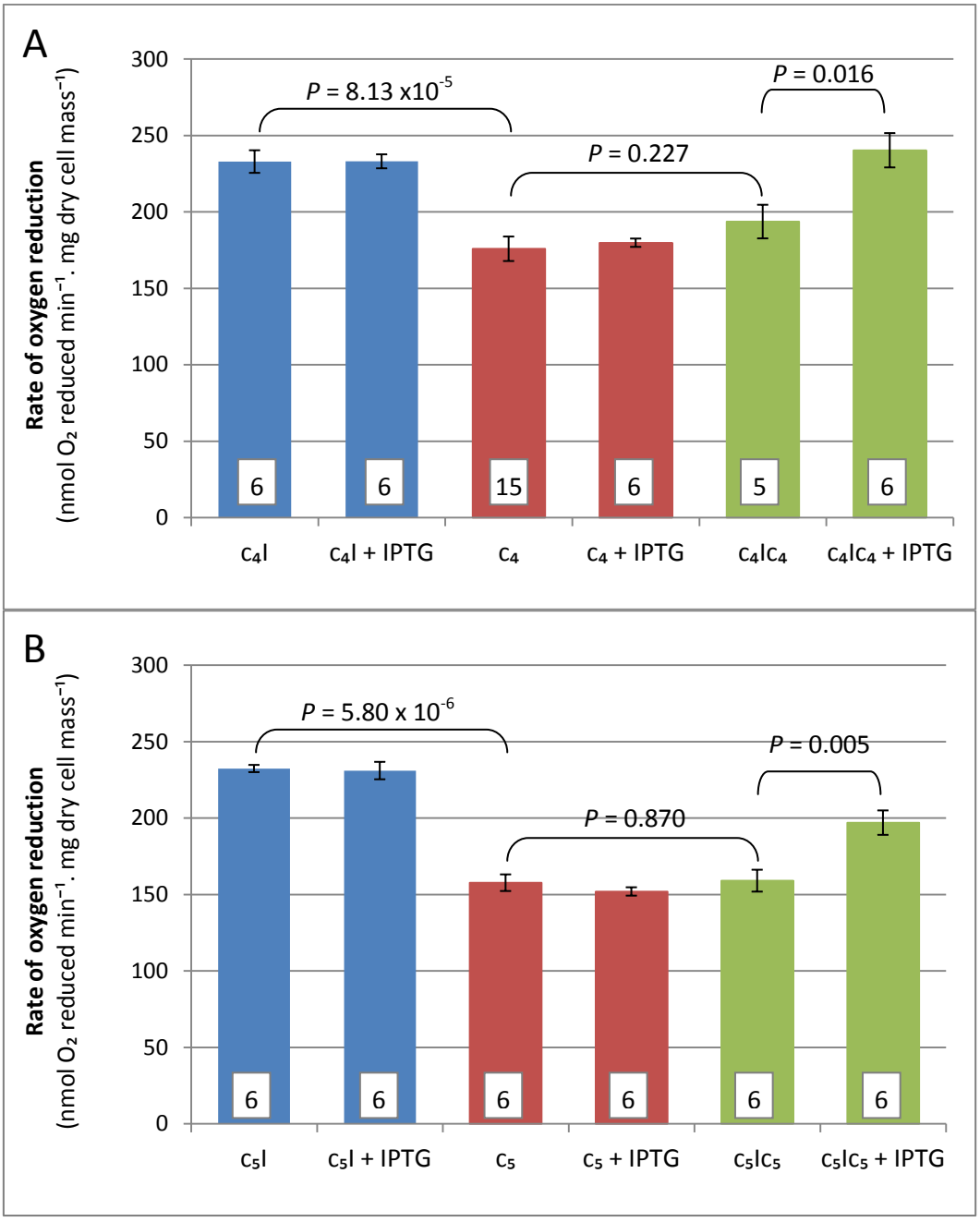
Figure 3.3 – SDS-PAGE gels stained for covalently bound heme in complemented cytochrome c_4 and c_5 deletion mutant strains

A) The presence of covalently bound heme in strains JCGC10 (labelled c_4I), JCGC801 (labelled Δc_4) and JCGC820 (labelled c_4I Δc_4) in the absence and presence of IPTG (indicated by – and + signs, respectively). Cultures were grown under oxygen-sufficient conditions and samples were taken after 7 hours of growth.

B) The presence of covalently bound heme in strains JCGC11 (labelled c_5I), JCGC860 (labelled Δc_5) and JCGC870 (labelled c_5I Δc_5) in the absence and presence of IPTG (indicated by – and + signs, respectively). Cultures were grown under oxygen-sufficient conditions and samples were taken after 7 hours of growth.

Figure 3.4 – Rates of oxygen reduction by washed bacterial suspensions of complemented cytochrome c_4 and c_5 deletion mutant strains

A) Rates of oxygen reduction by washed bacterial suspensions of strains JCGC10 (labelled c_4I), JCGC810 (labelled Δc_4) and JCGC820 (labelled c_4IΔc_4). Cultures were grown under oxygen-sufficient conditions in the presence or absence of 1 mM IPTG until the optical density at 650 nm reached a value between 0.8-1. Bacteria were then harvested, washed and their ability to reduce oxygen was measured using a Clark-type oxygen electrode. Rates are nmol of oxygen reduced min^{-1}. (mg of bacterial biomass) $^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.
B) Rates of oxygen reduction by washed bacterial suspensions of strains JCGC11 (labelled c_5I), JCGC860 (labelled Δc_5) and JCGC870 (labelled c_5IΔc_5). Bacteria were assayed as described above. Graphs are labelled as above.


A
Rate of oxygen reduction
(nmol O2 reduced min-1. mg dry cell mass-1)
300
250
200
150
100
50
0
P = 8.13 x10-5
P = 0.016
P = 0.227
6
6
15
6
5
6
c4I
c4I + IPTG
c4
c4 + IPTG
c4Ic4
c4Ic4 + IPTG
B
Rate of oxygen reduction
(nmol O2 reduced min-1. mg dry cell mass-1)
300
250
200
150
100
50
0
P = 5.80 x 10-6
P = 0.005
P = 0.870
6
6
6
6
6
6
c5I
c5I + IPTG
c5
c5 + IPTG
c5Ic5
c5Ic5 + IPTG

c_2 deletion mutation was transferred into cytochrome c_4 and c_5 mutants to create double mutants defective in cytochromes c_2 and c_4 (strain JCGC853), and cytochromes c_2 and c_5 (strain JCGC852), respectively. These strains were grown under oxygen-sufficient conditions, then harvested and their oxygen reduction rates determined. The rate of oxygen reduction by the cytochrome c_2-cytochrome c_4 double mutant in the presence of lactate was similar to that of the parent strain, indicating that the capacity of the cytochrome c_5 branch of the respiratory chain is sufficient to sustain a full rate of oxidation of physiological substrates (Table 3.1). Consistent with this conclusion, the rate of ascorbate and TMPD oxidation by this double mutant was only 15% less than that of the parent strain. In contrast, the rates of oxidation of both lactate and ascorbate plus TMPD by the double mutant defective in both cytochromes c_2 and c_5 were far lower than those of the other mutants, confirming that the capacity of the cytochrome c_4 branch of electron transfer to oxygen is lower than that of the cytochrome c_5 branch.

3.5 Failure to isolate a double mutant defective in both cytochromes c_4 and c_5

Various methods were used in attempts to construct a double mutant defective in both cytochromes c_4 and c_5, by both transforming a piliated cytochrome c_4 mutant with a cytochrome c_5 deletion-insertion cassette and vice versa. In more than 20 such attempts, *fnr* or other single mutants were isolated in parallel control experiments with the same recipient cultures, but no double mutants were isolated. Furthermore, attempts to recover double-mutant transformants anaerobically in the presence of nitrite were also unsuccessful, even when the recipient had been pre-adapted to oxygen-limited but nitrite-supplemented growth. This extensive series of experiments provided strong evidence that either

cytochrome c_4 or cytochrome c_5 is essential for gonococcal survival because they provide parallel pathways for electron transfer between the cytochrome bc_1 complex and cytochrome cbb_3 oxidase.

3.6 IPTG-dependent survival of a double mutant defective in both cytochromes c_4 and c_5 complemented by an inducible copy of the ectopically located *cycB* gene

A *cycA* mutation was transformed into strain JCGC870 in which the *cycB* mutation had been complemented by an ectopically expressed copy of *cycB* integrated at the *aspC-lctP* locus on the gonococcal chromosome. Transformants were created and maintained in the presence of IPTG and plated onto either unsupplemented GC agar or GC agar supplemented with 0.1 mM or 0.5 mM IPTG. Unlike strain JCGC870, which formed normal colonies on all three media, the survival of strain JCGC880 was totally dependent upon the presence of IPTG. Furthermore, the growth of this strain in GC broth stopped a few hours after subculture from the IPTG-supplemented inoculum as the cytochrome c_5 complement of the inoculum was diluted out and had stopped completely after 5 hours of growth (Figure 3.5). In contrast, growth in the presence of IPTG was indistinguishable from that of the *cycA* single mutant. These experiments confirmed that gonococcal growth and survival are totally dependent upon the expression of either cytochrome c_4 or cytochrome c_5. Furthermore, at least under the conditions normally used to culture gonococci in the laboratory, cytochrome c_2 cannot support growth or oxygen respiration.

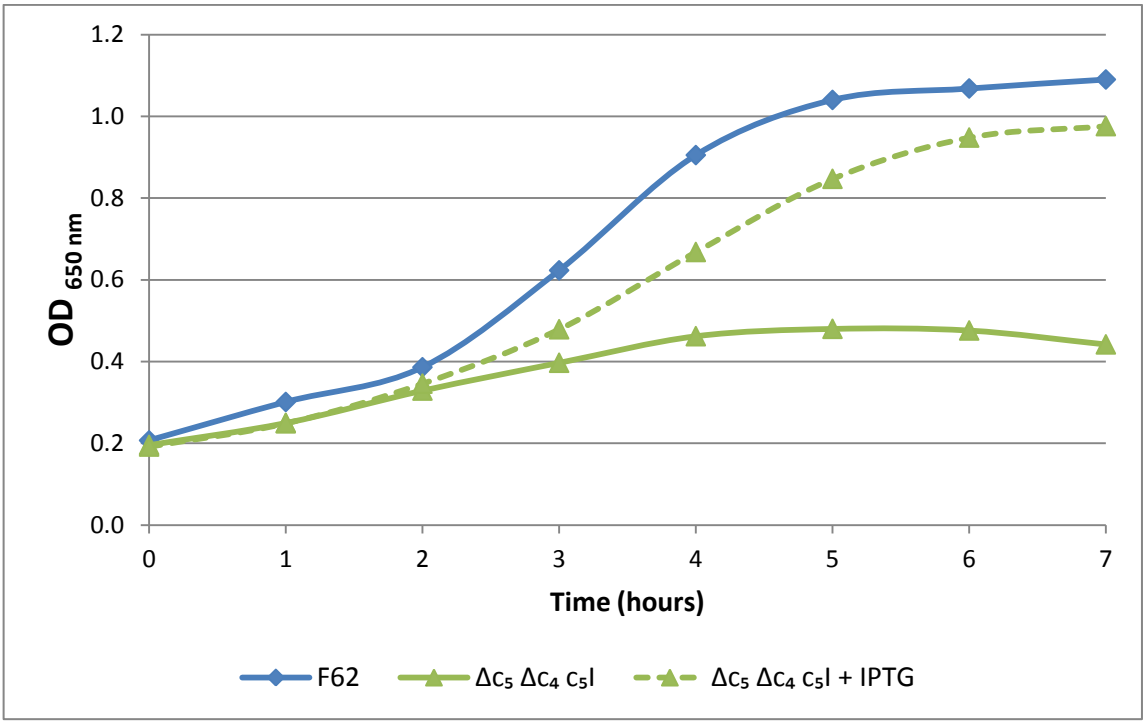


Figure 3.5 – Oxygen-sufficient growth of strains F62 and JCGC880 (Δ*c*$_5$ Δ*c*$_4$ *c*$_5$I) in the absence and presence of IPTG

Continuous lines indicate that no IPTG was added to cultures, whereas dotted lines indicate cultures where 0.1 mM IPTG was added immediately post-inoculation. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

3.7 Discussion

The cytochrome cbb_3 family of cytochrome oxidases is widely distributed among microaerobic bacteria, many of which also synthesize the diheme cytochromes c_4 and c_5 (reviewed in Pitcher & Watmough, 2004). There have been many studies of the distribution, assembly, mechanism of proton pumping, and biophysical properties of cytochrome cbb_3 oxidases from various bacteria, and the structure of the cytochrome cbb_3 oxidase from *Pseudomonas stutzeri* was recently published (Preisig *et al.*, 1993; García-Horsman *et al.*, 1994a; Gray *et al.*, 1994; de Gier *et al.*, 1996; Preisig *et al.*, 1996; Visser *et al.*, 1997; Arslan *et al.*, 2000; Pitcher & Watmough, 2004; Buschmann *et al.*, 2010). Although soluble monoheme cytochromes transfer electrons between the cytochrome bc_1 complex and cytochrome caa_3 oxidase, little is known about how electrons are transferred from the cytochrome bc_1 complex to the cytochrome cbb_3 oxidase family.

3.7.1 Essential roles of either cytochrome c_4 or cytochrome c_5 in electron transfer between the gonococcal cytochrome bc_1 complex and cytochrome oxidase

Downs and Jones were among the first groups to propose that in *Azotobacter vinelandii*, the diheme cytochrome c_4 and monoheme cytochrome c_5 might provide alternative but parallel pathways for electron transfer from the cytochrome bc_1 complex to the terminal oxidase (Downs & Jones, 1975). Much later, it was realized that there are multiple cytochrome oxidases in *A. vinelandii* and that one of them is a homologue of the *B. japonicum* high-affinity oxidase FixNOPQ (Kelly *et al.*, 1990; Kolonay *et al.*, 1994; Thöny-Meyer *et al.*, 1994; D'mello *et al.*, 1997). *A. vinelandii* single mutants defective in either cytochrome c_4 or

cytochrome c_5 have been constructed, and as in the current gonococcal study, rates of ascorbate-TMPD oxidation were lower in each of the mutants than in the parent strain. However, unlike the gonococcus, the loss of one or the other of these cytochromes had little effect on oxygen-sufficient growth or on rates of respiration in the presence of physiological substrates (Ng *et al.*, 1995; Rey & Maier, 1997). Also unlike the gonococcus was the successful isolation of a double mutant defective in both cytochromes c_4 and c_5 (Rey & Maier, 1997). This is highly significant because it is consistent with the presence of alternative respiratory pathways in *Azotobacter* that are unavailable to pathogenic *Neisseria*. This alone provides strong evidence not only that gonococcal cytochromes c_4 and c_5 are involved in electron transfer to oxygen but also that there is no major third pathway for oxygen reduction. This finding suggests that one of these two cytochromes is essential for survival when the gene for the other has been mutated, as shown in Figure 3.6. This conclusion is supported by the demonstration that both single mutants are more sensitive to high levels of aeration than the parent strain and that the accumulation of AniA, the product of an FNR-dependent gene, is delayed during oxygen-limited growth because the cytochrome c_4 and c_5 mutants are less able to reduce oxygen than the parent (Li *et al.*, 2010). This results in the delayed activation of the FNR protein because oxygen prevents the incorporation of the iron-sulfur centre of FNR, which in turn prevents dimerization and transcription activation (Overton *et al.*, 2003). Our conclusion that cytochromes c_4 and c_5 provide the only physiologically significant pathways for electron transfer to oxygen was confirmed by the demonstration that the survival of a *cycA cycB* double mutant was totally dependent upon the IPTG-induced expression of an ectopic copy of *cycB* (Figure 3.5).

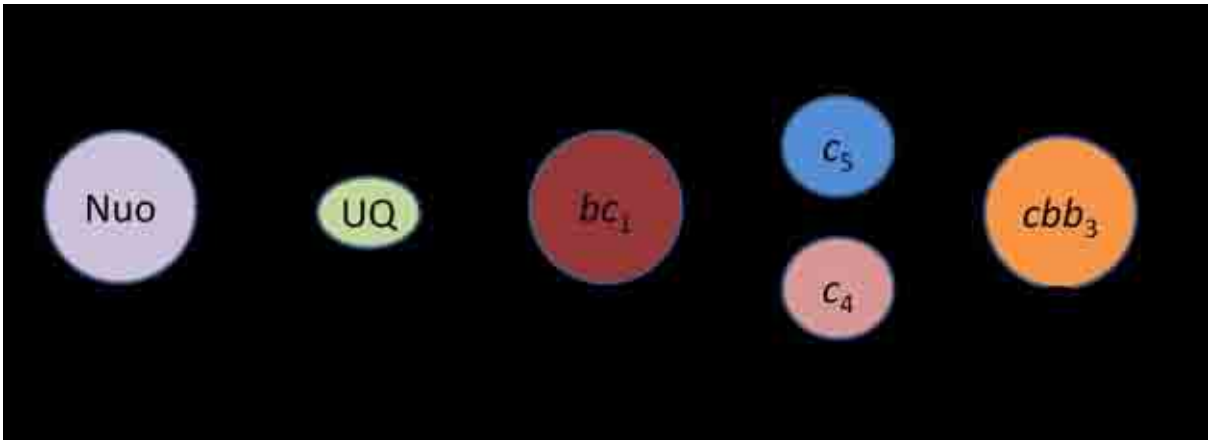


Figure 3.6 – Scheme for electron transfer from NADH to oxygen in *Neisseria gonorrhoeae*

Nuo, NADH dehydrogenase; UQ, ubiquinone; bc_1, cytochrome bc_1 complex; c_5, cytochrome c_5; c_4, cytochrome c_4; and cbb_3, cytochrome cbb_3 oxidase. Arrows represent electron transfer.

3.7.2 Lack of a significant role of cytochrome c_2 in electron transfer to cytochrome oxidase

Gonococcal cytochrome c_2 is clearly orthologous to cytochrome c_x of *N. meningitidis* (Deeudom *et al.*, 2006) but is only distantly related to any other *c*-type cytochromes currently in the databases. As previously noted (Deeudom *et al.*, 2006), residues 30 to 132 are 34% similar to cytochrome c_{552} of *Thermus thermophilus*, which was proposed to transfer electrons between the cytochrome bc_1 complex and cytochrome cbb_3 oxidase in that organism. As small, soluble, *c*-type cytochromes are normally involved in electron transfer between the cytochrome bc_1 complex and cytochrome oxidases of both mammals and many types of bacteria, cytochrome c_2 was a possible candidate to fulfil such a role in the gonococcus. However, in contrast to cytochromes c_4 and c_5, no evidence was found to show that cytochrome c_2 is involved directly in electron transfer to oxygen; on the contrary, oxygen reduction by the *cccA* mutant was more rapid than that of the parent strain, and this is supported by Western blotting data that showed a more rapid accumulation of AniA in oxygen-limited cultures of the *cccA* mutant than in cultures of the parent strain (Li *et al.*, 2010). Although the predicted native cytochrome has not until recently been seen on polyacrylamide gels stained for proteins with covalently bound heme, the evidence presented above indicates that the *cccA* gene is expressed. First, the *cccA* mutant reduced oxygen slightly more rapidly than the parent strain (Table 3.1). Second, clear differences in the effects of a secondary *cccA* mutation on mutants defective in *cycA* or *cycB* were detected (Table 3.1). The *cccA* mutation decreased the respiration rate of the *cycB* mutant, in which respiration was totally dependent upon cytochrome c_4, but increased the rate of oxygen reduction by the *cycA* mutant, in which oxygen reduction was dependent upon cytochrome

c_5 (Table 3.1).

3.7.3 Inefficient inhibition of respiration by myxothiazol

Myxothiazol was used in this study to determine whether the routes of electron transfer to the terminal oxidase were cytochrome bc_1 dependent. Although myxothiazol did decrease the rates of oxygen reduction by all strains tested, the concentration of myxothiazol used in these experiments should normally result in a much higher level of inhibition of respiration (N. Watmough, personal communication). Myxothiazol binds to the glutamic acid residue found in the PEWY amino acid motif of the cytochrome *b* subunit of the cytochrome bc_1 complex (e.g. Glu272 of *Gallus gallus* and Glu271 of bovine heart mitochondria), and this residue is highly conserved amongst mitochondrial cytochromes *b* (Degli Esposti *et al.*, 1993; Crofts, 2004; Esser *et al.*, 2004). Interestingly, in both *N. gonorrhoeae* and *N. meningitidis* a valine residue is found at this position instead of a glutamic acid residue (Figure 3.7). This could mean that myxothiazol might not bind very efficiently to the Neisserial cytochrome *b* of the cytochrome bc_1 complex, and could explain the decreased sensitivity of these bacteria to myxothiazol. A further possibility is that the concentration of cytochrome bc_1 found per cell may be higher in the gonococcus, which may mean that more myxothiazol would be required to inhibit all of the cytochrome bc_1 complexes present in each bacterium. Alternatively, it is possible that cytochromes c_4 and c_5 might be able to receive electrons from both the cytochrome bc_1 complex and the ubiquinol pool, the latter allowing the partial uncoupling of electron transfer to proton motive force. This could account for the lower level of inhibition of myxothiazol on respiration rates of the gonococcus. Unfortunately, the absence of an alternative oxidase in the gonococcus makes this hypothesis difficult to

Figure 3.7 – Multiple sequence alignment of the amino acid sequences of cytochromes *b* from the cytochrome bc_1 complexes of *N. gonorrhoeae*, *N. meningitidis* and *Gallus gallus*.

NG refers to *N. gonorrhoeae*, NM refers to *N. meningitidis* and GG refers to *Gallus gallus*. The relevant Glu and Val residues are highlighted. Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

investigate.

3.7.4 Oxygen sensitivity during growth of single and double mutants defective in cytochromes c_2, c_4, or c_5

Differences in the oxygen reduction rates of single and double mutants defective in *c*-type cytochromes c_2, c_4 and c_5 are reflected and corroborated by growth data reported in Li *et al.*, 2010. The oxygen sensitivity of single and double mutants defective in cytochromes c_2, c_4, or c_5 was determined by exposing gonococci to varying levels of aeration. Aerobic, oxygen-sufficient and oxygen-limited conditions were provided by growing the bacteria in cultures containing 10 mL, 30 mL and 60 mL of media, respectively. The growth of cytochrome c_4 and c_5 single mutants was found to be inhibited in highly aerated cultures compared to the parent strain and the cytochrome c_2 deletion mutant, which grew similarly. However, under oxygen-limited conditions the growth of all single mutants was indistinguishable from that of the parent strain. It was suggested that cytochromes c_4 and c_5, but not cytochrome c_2, might be important for electron transfer to the terminal oxidase. Double mutants defective in cytochromes c_2 and c_4, and cytochromes c_2 and c_5 were also grown under the same conditions with varying levels of aeration. Both strains were more sensitive to aeration than the parent strain, but the cytochrome c_2 c_4 double mutant was less sensitive to oxygen than the cytochrome c_4 mutant, and the cytochrome c_2 c_5 double mutant was more sensitive to oxygen than the cytochrome c_5 mutant (Li *et al.*, 2010). These observations suggest that cytochrome c_2 might regulate electron flow between the cytochrome c_4 and cytochrome c_5 branches of the respiratory chain. However, other explanations need to be investigated. It is possible that cytochrome c_2 could play a physiologically significant role in transferring

electrons to outer membrane redox proteins, such as the lipid-associated azurin, Laz, and the cytochrome *c* peroxidase Ccp, that protect gonococci against hydrogen peroxide (Trees & Spinola, 1990; Turner *et al.*, 2003; Seib *et al.*, 2004).

3.7.5 Why is a pathogen with a microaerobic lifestyle such a prolific source of electron transfer components?

To establish an infection, pathogens must be able to protect themselves against reactive oxygen and nitrogen species generated externally as part of the host defence mechanisms and by other bacteria that coinhabit their environment. The outer membrane lipoproteins Ccp, cytochrome *c*′, Laz, and AniA provide pathogenic *Neisseria* with a first line of defence against reactive oxygen and reactive nitrogen species generated by their human host (Turner *et al.*, 2003; Turner *et al.*, 2005). Results from this chapter with the single mutants defective in either cytochrome c_4 or c_5 have provided both insight into the rate-determining steps in gonococcal respiration and an explanation for the following long-standing paradox: why should an obligate human pathogen with a microaerobic lifestyle maintain such high rates of respiration and be such a prolific source of *c*-type cytochromes?

Rates of ascorbate-plus-TMPD-dependent respiration by washed bacterial suspensions of all of the strains studied were far higher (5.1- to 6.5-fold) than rates in the presence of the physiological substrate lactate (Table 3.1). This finding implies that the capacity of electron transfer between lactate and the cytochrome bc_1 complex is less than that of the terminal components of the respiratory chain, a conclusion supported by the fact that the sum of the rates of the TMPD oxidation of the *cycA* and *cycB* mutants was greater than that of the

parent strain. Despite the small loss of the respiratory capacity, the *cycA* and *cycB* single mutants were far more sensitive to excess oxygen than the parent (Li *et al.*, 2010), indicating that this excess capacity for oxygen reduction protects the gonococcus from reactive oxygen species generated during aerobic growth. One possible explanation for this finding is that one or both of these cytochromes are required to protect gonococci against reactive oxygen species generated during aerobic growth. It is more likely, however, that the increased sensitivity to oxygen is due to the increased generation of reactive oxygen species by the mutants, for example, by the chemical interaction of oxygen with reduced flavoproteins or the quinone pool (Hägerhäll *et al.*, 1999; Storz & Imlay, 1999), which will be more reduced in the *cycA* and *cycB* mutants than in the parent strain due to their lower rates of electron transfer from the cytochrome bc_1 complex to oxygen. If this is correct, the high respiratory capacity of gonococci prevents the accumulation of oxygen, thus minimising the generation of toxic oxygen species such as hydrogen peroxide, superoxide, and hydroxyl radicals. It is therefore proposed that the excess capacity for oxygen reduction provides a defence against reactive oxygen species generated as by-products of their own metabolism, which supplements the many other defence mechanisms documented previously (Seib *et al.*, 2006).

Chapter 4

The enigmatic cytochrome c_2

4.1 Introduction

The *cccA* gene (NG0292) encodes cytochrome c_2, a small protein with a predicted molecular mass of 16.5 kDa. It contains a C-X-X-C-H amino acid motif, suggesting that this protein binds heme covalently. As described in the previous chapter, gonococcal cytochrome c_2 does not appear to be oxidised by the cytochrome cbb_3 oxidase, so its function in the electron transfer chain of *Neisseria gonorrhoeae* remains unknown. For this reason, its potential role as an electron donor to AniA was explored.

4.2 Bioinformatic analysis of the gonococcal *cccA* gene

A BLAST search using the NG0292 sequence, excluding hits from the *Neisseria* genus, showed that cytochrome c_2 is similar to a domain of many copper-containing nitrite reductases. For example, cytochrome c_2 is 46% identical to the nitrite reductase of *Kangiella koreensis* and 46% identical to the nitrite reductase found in *Bdellovibrio bacteriovorus*. More interestingly, residues 30-132 of gonococcal cytochrome c_2 are 61% similar to residues 398-500 of the nitrite reductase AniA of *Moraxella catarrhalis* strain 46P47B1. *Moraxella catarrhalis* was for many years classified as a member of the *Neissera* genus, due to its similar morphology (Catlin, 1991). It is now known to belong to the gammaproteobacteria rather than the betaproteobacteria, the class to which the *Neisseria* genus belongs. Although *M. catarrhalis* has a nitrate reductase (Wang *et al.*, 2011), the rest of its denitrification pathway is similar to the pathogenic *Neisseria* as it encodes a nitrite reductase, AniA and a nitric oxide reductase, NorB. A sequence alignment of the *M. catarrhalis* and *N. gonorrhoeae* AniA proteins shows that they share 63% sequence similarity (Figure 4.1). However, the *M.*

```
Mc AniA       1 mskptlikttlicalsalmlsgcsnqadkaaqpksstvdaaakta-nadn     49
                |.:..|  ..:|.:|.||...|    .:.|||..:.|..|:|:.| :|..
Ng AniA       1 MKRQAL--AAMIASLFALAACG----GEPAAQAPAETPAASAEAASSAAQ     44

Mc AniA      50 aasqehqgelpvidaivthapevpppvdrdhpakvvvkmetvekvmrlad     99
                |.::...||||||||:.||||||||.:|||:||||.||||||||.|::.|
Ng AniA      45 ATAETPAGELPVIDAVTTHAPEVPPAIDRDYPAKVRVKMETVEKTMKMDD     94

Mc AniA     100 gveyqfwtfggqvpgqmirvregdtievqfsnhpdskmphnvdfhaatgp    149
                ||||::|||.|.|||:||||||||:||:|||:|.|.:||||||||||.
Ng AniA      95 GVEYRYWTFDGDVPGRMIRVREGDTVEVEFSNNPSSTVPHNVDFHAATGQ    144

Mc AniA     150 gggaeasftapghtstfsfkalqpglyvyhcavapvgmhiangmyglilv    199
                ||||.|:|||||.|||||||||||||:||||||||||||||||||||||
Ng AniA     145 GGGAAATFTAPGRTSTFSFKALQPGLYIYHCAVAPVGMHIANGMYGLILV    194

Mc AniA     200 epkeglpkvdkeyyvmqgdfytkgkygeqglqpfdmekairedaeyvvfn    249
                ||||||||||||:|::||||||||||.|.||||||||:||:.|..||||||
Ng AniA     195 EPKEGLPKVDKEFYIVQGDFYTKGKKGAQGLQPFDMDKAVAEQPEYVVFN    244

Mc AniA     250 gsvgaltgenalkakvgetvrlfvgnggpnltssfhvigeifdkvhfegg    299
                |.|||:.|:||||||.||||||::||||||||.|||||||||||||:.|||
Ng AniA     245 GHVGAIAGDNALKAKAGETVRMYVGNGGPNLVSSFHVIGEIFDKVYVEGG    294

Mc AniA     300 kgenhniqttlipaggaaitefkvdvpgdyvlvdhaifrafnkgalgilk    349
                |..|.|:|:|::||||:||.|||||:||:|.||||:||||||||||.||
Ng AniA     295 KLINENVQSTIVPAGGSAIVEFKVDIPGNYTLVDHSIFRAFNKGALGQLK    344

Mc AniA     350 vegeenheiyshkqtdavylpegapqaidtqeapktpapanlqeqikagk    399
                |||.||.||.:.|.:|..|...||..| ....||...|||....:    |
Ng AniA     345 VEGAENPEIMTQKLSDTAYAGSGAASA-PAASAPAASAPAASASE----K    389

Mc AniA     400 atydsncaachqpdgkgvpnafpplansdylnadharaasivanglsgki    449
                :.|
Ng AniA     390 SVY-------------------------------------------------    392

Mc AniA     450 tvngnqyesvmpaialsdqqianvitytlnsfgnkggqlsaddvakakkt    499

Ng AniA     393 -------------------------------------------------    392

Mc AniA     500 kpn    502

Ng AniA     393 ---    392
```

Figure 4.1 – Pairwise alignment of the amino acid sequence of AniA proteins from *Moraxella catarrhalis* and *Neisseria gonorrhoeae*

Moraxella catarrhalis AniA amino acid sequence is labelled as *Mc* AniA, and the *Neisseria gonorrhoeae* AniA amino acid sequence is labelled as *Ng* AniA. Vertical lines (|) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

catarrhalis protein is larger than the gonococcal AniA protein, with an extension of about 100 amino acids. Interestingly, this extension of amino acids in the *M. catarrhalis* AniA is 61% similar to the gonococcal cytochrome c_2. This suggests that the AniA protein of *M. catarrhalis* has an extra *C*-terminal domain containing a heme-binding site. One could speculate that perhaps cytochrome c_2 fulfils the role of the heme-binding domain in the gonococcal AniA protein.

A pairwise alignment comparing a hypothetical hybrid of gonococcal AniA and cytochrome c_2 proteins (containing all AniA residues plus residues 30-132 of cytochrome c_2) with the *M. catarrhalis* AniA protein shows that the two proteins are 72.1% similar and 60.6% identical (Figure 4.2). There is also similarity between the AniA-cytochrome c_2 hybrid and the copper-containing nitrite reductases of a small group of bacteria, including *Pseudoalteromonas haloplanktis* TAC125 (a member of the gammaproteobacteria), *Bdellovibrio bacteriovorus* HD100 (a member of the deltaproteobacteria), *Psychrobacter sp.* 1501 (a member of the gammaproteobacteria) and *Kingella kingae* ATCC23330 (a member of the betaproteobacteria). A multiple alignment highlighting these similarities is shown in Figure 4.3. The AniA protein from *Pseudoalteromonas haloplanktis* TAC125 is 64.5% similar to the gonococcal AniA-c_2 hybrid. The crystal structures of AniA from *P. haloplanktis* (PDB entry 2ZOO) and *N. gonorrhoeae* (PDB entry 1KBW) have been published (Boulanger & Murphy, 2002). A comparison of their trimeric forms revealed that they are homologous in their larger, copper-containing domains (Figure 4.4). However, if like the gonococcal AniA protein the *P. haloplanktis* AniA was localised on the outer membrane, the presence of the extra heme-binding domain at the bottom of the *P. haloplanktis* AniA protein would increase the

```
Mc AniA       1 mskptlikttlicalsalmlsgcsnqadkaaqpksstvdaaakta-nadn     49
                |.:..|  ..:|.:|.||...|    .:.|||..:.|..|:|:.| :|..
Ng AniA+c2    1 MKRQAL--AAMIASLFALAACG----GEPAAQAPAETPAASAEAASSAAQ     44

Mc AniA      50 aasqehqgelpvidaivthapevpppvdrdhpakvvvkmetvekvmrlad     99
                |.::...||||||||:.||||||||.:|||:||||.||||||||.|::.|
Ng AniA+c2   45 ATAETPAGELPVIDAVTTHAPEVPPAIDRDYPAKVRVKMETVEKTMKMDD     94

Mc AniA     100 gveyqfwtfggqvpgqmirvregdtievqfsnhpdskmphnvdfhaatgp    149
                ||||::|||.|.|||:||||||||:||:|||:|.|.:||||||||||.
Ng AniA+c2   95 GVEYRYWTFDGDVPGRMIRVREGDTVEVEFSNNPSSTVPHNVDFHAATGQ    144

Mc AniA     150 gggaeasftapghtstfsfkalqpglyvyhcavapvgmhiangmyglilv    199
                ||||.|:|||||.|||||||||||||:||||||||||||||||||||||
Ng AniA+c2  145 GGGAAATFTAPGRTSTFSFKALQPGLYIYHCAVAPVGMHIANGMYGLILV    194

Mc AniA     200 epkeglpkvdkeyyvmqgdfytkgkygeqglqpfdmekairedaeyvvfn    249
                |||||||||||:|::|||||||||.|.|||||||:||:.|..||||||
Ng AniA+c2  195 EPKEGLPKVDKEFYIVQGDFYTKGKKGAQGLQPFDMDKAVAEQPEYVVFN    244

Mc AniA     250 gsvgaltgenalkakvgetvrlfvgnggpnltssfhvigeifdkvhfegg    299
                |.|||:.|:||||||.||||||::||||||||.|||||||||||||:.|||
Ng AniA+c2  245 GHVGAIAGDNALKAKAGETVRMYVGNGGPNLVSSFHVIGEIFDKVYVEGG    294

Mc AniA     300 kgenhniqttlipaggaaitefkvdvpgdyvlvdhaifrafnkgalgilk    349
                |..|.|:|:|::||||:||.|||||:||:|.||||:||||||||||.||
Ng AniA+c2  295 KLINENVQSTIVPAGGSAIVEFKVDIPGNYTLVDHSIFRAFNKGALGQLK    344

Mc AniA     350 vegeenheiyshkqtdavylpegapqaidtqeapktpap-anlqeqikag    398
                |||.||.||.:.|.:|..|...||..| ....||...|| |:..|:...|
Ng AniA+c2  345 VEGAENPEIMTQKLSDTAYAGSGAASA-PAASAPAASAPAASASEKSVYG    393

Mc AniA     399 katydsncaachqpdgkgvpnafpplansdy-lnadharaasivanglsg    447
                :..|:|||.|||...|:|...||||..||| :|..|....|:| .|::|
Ng AniA+c2  394 QKVYESNCIACHGKKGEGRGTAFPPLFRSDYIMNKPHVLLHSMV-KGING    442

Mc AniA     448 kitvngnqyesvmpaialsdqqianvitytlnsfgnkggqlsaddv--ak    495
                .|.|||..|...|||.|:||..||.|.||.:|:|.|.||.::..||  ||
Ng AniA+c2  443 TIKVNGKTYNGFMPATAISDADIAAVATYIMNAFDNGGGSVTEKDVKQAK    492

Mc AniA     496 akktkpn------------------    502
                .||.:.:
Ng AniA+c2  493 GKKNQTDKMPSETGNPASDGIQIKPF    518
```

Figure 4.2 – Pairwise alignment of the amino acid sequence of the AniA protein from *Moraxella catarrhalis* and the AniA-cytochrome c_2 hybrid of *Neisseria gonorrhoeae*

Moraxella catarrhalis AniA amino acid sequence is labelled as *Mc* AniA, and the *Neisseria gonorrhoeae* AniA-cytochrome c_2 hybrid amino acid sequence is labelled as *Ng* AniA+c2. The AniA-cytochrome c_2 hybrid contains the full amino acid sequence of the AniA protein followed by residues 30-132 of cytochrome c_2 (the latter indicated by underlined residues). Vertical lines (|) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

Figure 4.3 – Multiple sequence alignment of the amino acid sequence of the AniA proteins from *Pseudoalteromonas haloplanktis* TAC125, *Bdellovibrio bacteriovorus* HD100, *Moraxella catarrhalis*, *Psychrobacter sp.* 1501, *Kingella kingae* ATCC23330 and the AniA-cytochrome c_2 hybrid of *Neisseria gonorrhoeae*

Phalo is *Pseudoalteromonas haloplanktis* TAC125; Bbac is *Bdellovibrio bacteriovorus* HD100; Mcat is *Moraxella catarrhalis;* Psp is *Psychrobacter sp.* 1501; Kkin is *Kingella kingae* ATCC23330; and Ngon is the AniA-cytochrome c_2 hybrid of *Neisseria gonorrhoeae*. The AniA-cytochrome c_2 hybrid contains the full amino acid sequence of the AniA protein followed by residues 30-132 of cytochrome c_2 (the latter indicated by underlined residues). Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.

```
Phalo      ---------MKIQPNKHTLWLPLIAILFSGNLLAASNLKTE------------------- 32
Bbac       ---------MKDWNYKRLLLTGAIACLAPMPVLADNIKGEE------------------- 32
Mcat       --------MSKPTLIKTTLICALSALMLSGCSNQADKAAQPKSSTVDAAAKTANADNAAS 52
Psp        MLISNLQSSKKPHLYSKLALACLLAMGTVGLVGCDDKAADN--DTDQAASTATNESAQHD 58
Kkin       ---MKICTLAALIASAFALTACQQPQTSAKPAEPASTKPAETTAASSPAQAAPAQAASEP 57
Ngon       ----------MKRQALAAMIASLFALAACGGEPAAQAPAETP---AASAEAASSAAQATA 47
                                .         .

Phalo      ----------QAILTPPPMVPPAINRDHSAKVVINLETREQVGRIADGVEYVFWSFGETV 82
Bbac       ----------VAVLTDAPEVPPPITRKHATKVIVNLETKEVKLRLADGVDYTFWTFGGKV 82
Mcat       QEHQGELPVIDAIVTHAPEVPPPVDRDHPAKVVVKMETVEKVMRLADGVEYQFWTFGGQV 112
Psp        STAKGELPVIDAILTSAPEVPPPIDRDYPAKVVVKMETVEKVMRLADGVEYNFWTFDGTV 118
Kkin       EVAAADLPVIDAVLTHAPEAPPPVNRNHAARVKVKIEIQEKVMRMADGVEYKFWTFGGQV 117
Ngon       ETPAGELPVIDAVTTHAPEVPPAIDRDYPAKVRVKMETVEKTMKMDDGVEYRYWTFDGDV 107
                     *: * .* .**.: *.:.::* :::*  *   :: ***:* :*:*.  *

Phalo      PGSFIRVREGDEIEFNLSNHPSSKMPHNIDLHAVTGPGGGAESSFTAPGHTSTFNFKALN 142
Bbac       PGKFIRIREGDQVEFHLHNHPSSKLPHNIDLHAVTGQGGGAEGSFTAPGHSSTFSFKALN 142
Mcat       PGQMIRVREGDTIEVQFSNHPDSKMPHNVDFHAATGPGGGAEASFTAPGHTSTFSFKALQ 172
Psp        PGSFIRVREGDEIEFHLSNHPDSKMPHNIDLHAVTGPGGGAASSFTSPGHTSEFNFTALK 178
Kkin       PGQMIRVREGDIVDVEFANRSDSTVPHNIDFHAATGPGGGAEASFTAPGHVSNFSFKALQ 177
Ngon       PGRMIRVREGDTVEVEFSNNPSSTVPHNVDFHAATGQGGGAAATFTAPGRTSTFSFKALQ 167
           ** :**:**** ::..: *...*.:***:*:**.** **** .:**:**: * *.*.**:

Phalo      PGLYIYHCATAPVGMHIANGMYGLILVEPKEGLAPVDREYYLVQGDFYTKGEFGEAGLQP 202
Bbac       PGLYVYHCATAPVGMHIANGMYGLILVEPKEGLPKVDREFYVLQSEFYTKGKYGAPGLQP 202
Mcat       PGLYVYHCAVAPVGMHIANGMYGLILVEPKEGLPKVDKEYYVMQGDFYTKGKYGEQGLQP 232
Psp        PGLYVYHCAVAPVGMHIANGMYGLILVEPKEGLPKVDKEFYVMQGDFYTKGDYGDKGLQP 238
Kkin       PGLYIYHCATAPVGMHIANGMYGLILVEPKEGLPPVDKEFYVVQGDFYTKGKYGEAGLQP 237
Ngon       PGLYIYHCAVAPVGMHIANGMYGLILVEPKEGLPKVDKEFYIVQGDFYTKGKKGAQGLQP 227
           ****:****.**********************. **:*:*::*.:*****. *  ****

Phalo      FDMAKAIDEDADYVVFNGSVGSTTDENSLTAKVGETVRLYIGNGGPNLVSSFHVIGEIFD 262
Bbac       FSMTKAVEEKADYVVFNGSVGSLVGDNAMKAKTGEKVRLFVGNGGPNLVSSFHVIGEIFD 262
Mcat       FDMEKAIREDAEYVVFNGSVGALTGENALKAKVGETVRLFVGNGGPNLTSSFHVIGEIFD 292
Psp        FDMEKAVKEDADYVVFNGSVGSLTGDNSLKAKVGETVRLFVGNGGPNLISSFHVIGEIFD 298
Kkin       FDMEKAIREQPDYVVFNGNVGSIAGDNALKAKVGDKIRLFVGNGGPNLVSSFHVIGEIFD 297
Ngon       FDMDKAVAEQPEYVVFNGHVGAIAGDNALKAKAGETVRMYVGNGGPNLVSSFHVIGEIFD 287
           *.* **: *..:****** **: ..:*::.**.*:.:*:::******* ***********

Phalo      TVYVEGGSLKNHNVQTTLIPAGGAAIVEFKVEVPGTFILVDHSIFRAFNKGALAMLKVEG 322
Bbac       KVYVEGGKLVNENVQTTLIPAGGSAIVEFKLDTTGTFILVDHSIFRAFNKGAIGMLKVEG 322
Mcat       KVHFEGGKGENHNIQTTLIPAGGAAITEFKVDVPGDYVLVDHAIFRAFNKGALGILKVEG 352
Psp        KVNIEGGLTENHNVQTTLIPAGGAAITEFKLDVPGEYVMVDHSIFRAFNKGALGILAVDG 358
Kkin       TVYVEGGALKNHNVQTTLVPAGGAAIVDFKVDVPGTYNLVDHSIFRTFNKGALGQIKVEG 357
Ngon       KVYVEGGKLINENVQSTIVPAGGSAIVEFKVDIPGNYTLVDHSIFRAFNKGALGQLKVEG 347
           .* .***   *.*:*:*::****:**.:**:: .* : :***:***:*****:. : *:*

Phalo      PDDHSIFTGKTAENVYLPEGSAIQSLD------NTFTK--ITANNKDEQIRFGQRVYEAN 374
Bbac       KEDHEVYSGKTKDGIYLPEGGVIQEIG------AEAPKQ-IPAKTLEERLSGGKRIYESS 375
Mcat       EENHEIYSHKQTDAVYLPEG-APQAIDT-----QEAPKTPAPAN-LQEQIKAGKATYDSN 405
Psp        PEDKKIYSGKVREQVYLPEGSAAQSIGN-----SNTPKPKVTAKNLQQRIKMGESVYASN 413
Kkin       EENKDIFSGKLTEGIYHSEGGAIQTAPLTASETAKVAAGPATASSKEDRIKLGETVYKAN 417
Ngon       AENPEIMTQKLSDTAYAGSGAASAPAAS-------APAASAPAASASEKSVYGQKVYESN 400
            :: .: : *  :  *  .* .              .    .*   .::   *:  * :.

Phalo      CMACHQANGEGIPGAFPPLAKSDYLNNNPLLGVNAIIKGLSGPIKVNNVNYNGVMPAMNL 434
Bbac       CFACHQSNGQGLPGAFPPLAKSDFLKNK-DKAISAVIHGLEGPIKVNGKEYNSVMPAQIL 434
Mcat       CAACHQPDGKGVPNAFPPLANSDYLNADHARAASIVANGLSGKITVNGNQYESVMPAIAL 465
Psp        CAACHQPNGTGVPNAFPPLAKSDYLNADPNRAIDAIHNGLSGKITVNGKEYNSVMPAVNL 473
Kkin       CLACHGVEGKGVEGAFPPLAESDYLNADYKRGIQAVVKGLSGEITVNGKKFNSVMPAVAL 477
Ngon       CIACHGKKGEGRGTAFPPLFRSDYIMNKPHVLLHSMVKGINGTIKVNGKTYNGFMPATAI 460
           * ***  .* *   ***** .**::  .       : :*:.* *.**.  ::..***  :

Phalo      NDEDIANVITFVLNNWDNAGGKVSAEQVAKQRK------------------------- 467
Bbac       SDEDAANVLTYIYSMWGNSKKVVTPADVKAVRAAGKK---------------------- 471
Mcat       SDQQIANVITYTLNSFGNKGGQLSADDVAKAKKNQAKLILKFN---------------- 508
Psp        DDEKTANVVTYILNSWGNKGGEITPEDVAARK------------------------- 505
Kkin       NDEDAANVLTFVLNSFGNKGGEVKPEEIAAARK------------------------- 510
Ngon       SDADIAAVATYIMNAFDNGGGSVTEKDVKQAKGKKNQTDKMPSETGNPASDGIQIKPF 518
           .* . * * *:  . :.*    :.  ::   :
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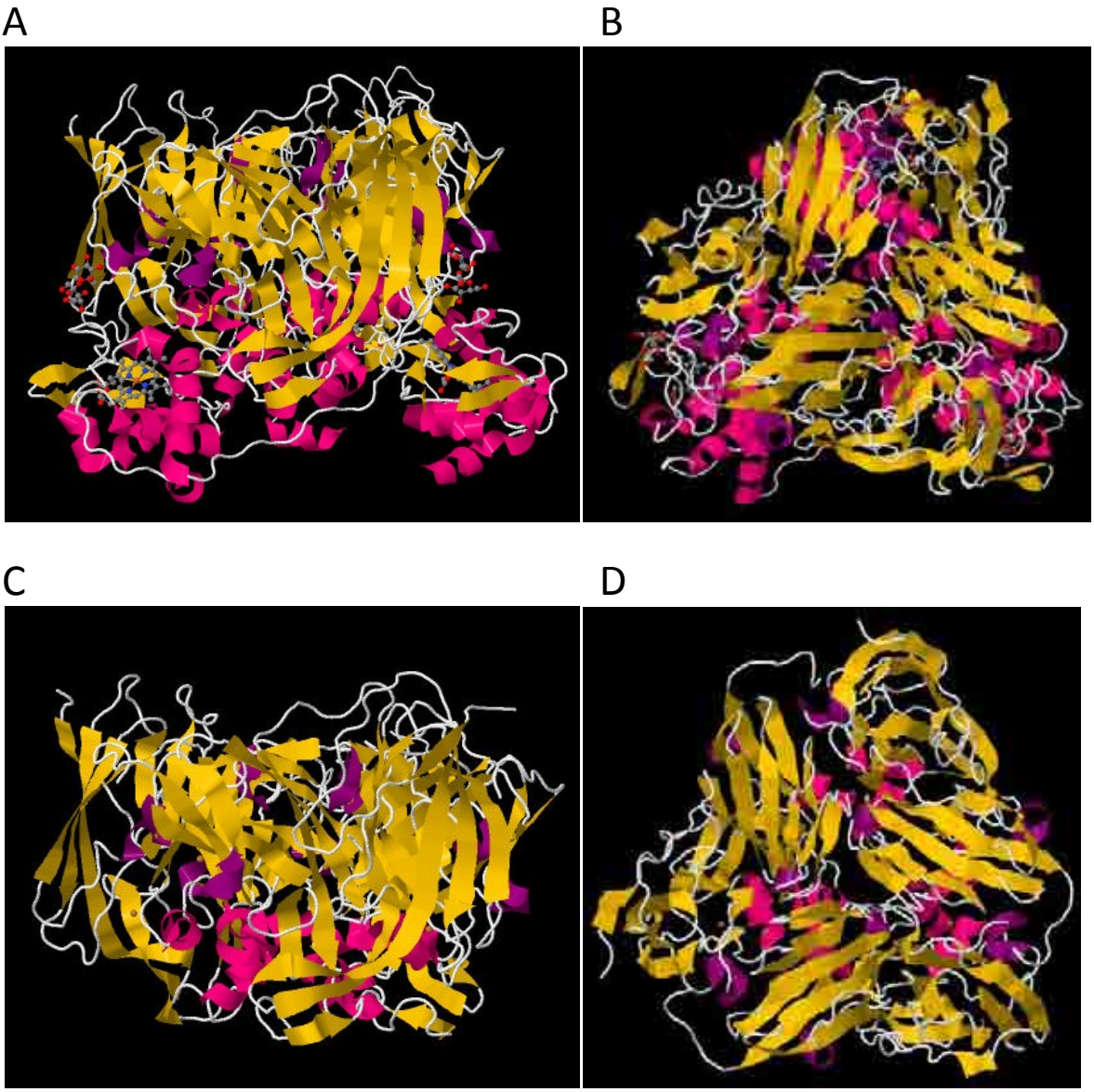


Figure 4.4 – Trimeric forms of AniA proteins from *Pseudoalteromonas haloplanktis* TAC125 and *Neisseria gonorrhoeae*

Trimeric forms of AniA proteins from *Pseudoalteromonas haloplanktis* TAC125 (panels A and B) and *Neisseria gonorrhoeae* (panels C and D). The *P. Haloplanktis* TAC125 structure is modified from PDB entry 2ZOO (Nojiri *et al.*, unpublished), and is shown from the side (panel A) and from the top (panel B). The *N. gonorrhoeae* structure is modified from PDB entry 1KBW (Boulanger & Murphy, 2002), and is shown from the side (panel C) and from the top (panel D).

size of the protein, potentially extending it further into the periplasm. The structure of the *P. haloplanktis* AniA monomer can be superimposed successfully onto the *N. gonorrhoeae* AniA monomer (Figure 4.5). The similarity between the two structures is high, except for the additional heme-binding domain found in the *P. haloplanktis* AniA. The high degree of similarity between cytochrome c_2 and the *C*-termini of the aforementioned nitrite reductases is suggestive of a role for cytochrome c_2 as a subunit of the gonococcal AniA complex. The potential association of cytochrome c_2 with the gonococcal AniA protein would hypothetically allow the gonococcal AniA to protrude further into the periplasm from the outer membrane, thus allowing more efficient electron transfer from the inner membrane redox proteins to AniA. The remainder of this chapter will attempt to establish the role of cytochrome c_2 in the electron transfer chain of the gonococcus.

4.3 Nitrite supplements oxygen-limited growth of gonococci

When oxygen is in short supply, gonococci can use nitrite as an alternative electron acceptor (Knapp & Clark, 1984). To determine whether nitrite could improve the growth rate of gonococci under oxygen-limited conditions, the parental F62 strain was grown under oxygen-limited growth conditions, as described in Chapter 2. It is clear from the growth data that the presence of nitrite increases the growth rate of the parent strain F62 under oxygen-limited conditions (Figure 4.6).

4.4 Cytochrome c_2 as a potential electron donor to AniA

As cytochrome c_2 was not shown to be an electron donor to the terminal oxidase, it was

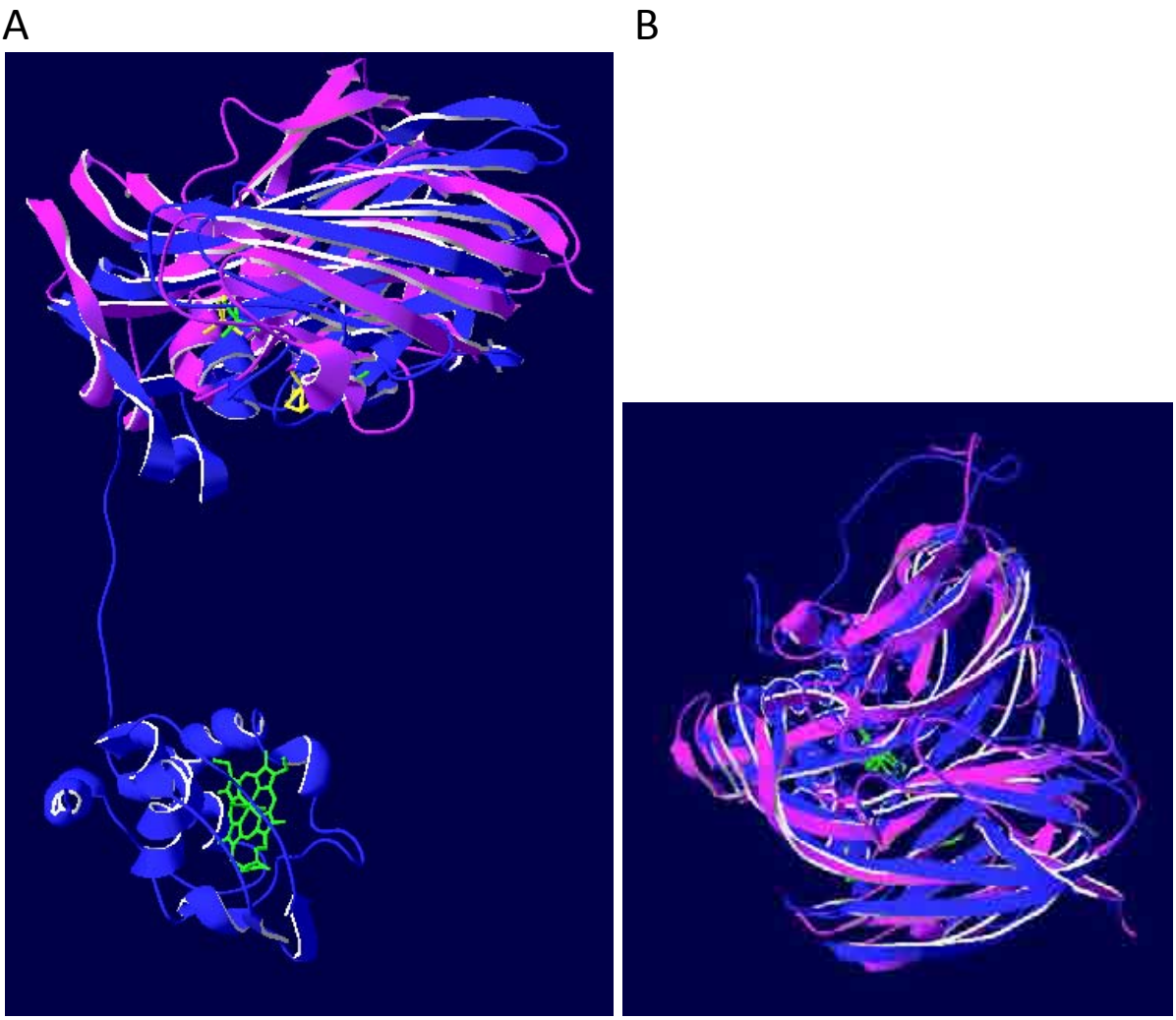


Figure 4.5 – Superimposition of monomeric forms of AniA from *Pseudoalteromonas haloplanktis* TAC125 (shown in blue) and *Neisseria gonorrhoeae* (shown in purple)

Pseudoalteromonas haloplanktis TAC125 AniA is shown in blue, *Neisseria gonorrhoeae* AniA is shown in purple. Panel (A) shows a side view, and panel (B) shows the structure viewed from above. Prosthetic groups from *P. haloplanktis* are shown in green, and from *N. gonorrhoeae* these groups are shown in yellow. Modified from PDB entries 1KBW (Boulanger & Murphy, 2002) and 2ZOO (Nojiri *et al.*, unpublished) using the Swiss-PdbViewer program (Guex & Peitsch, 1997).

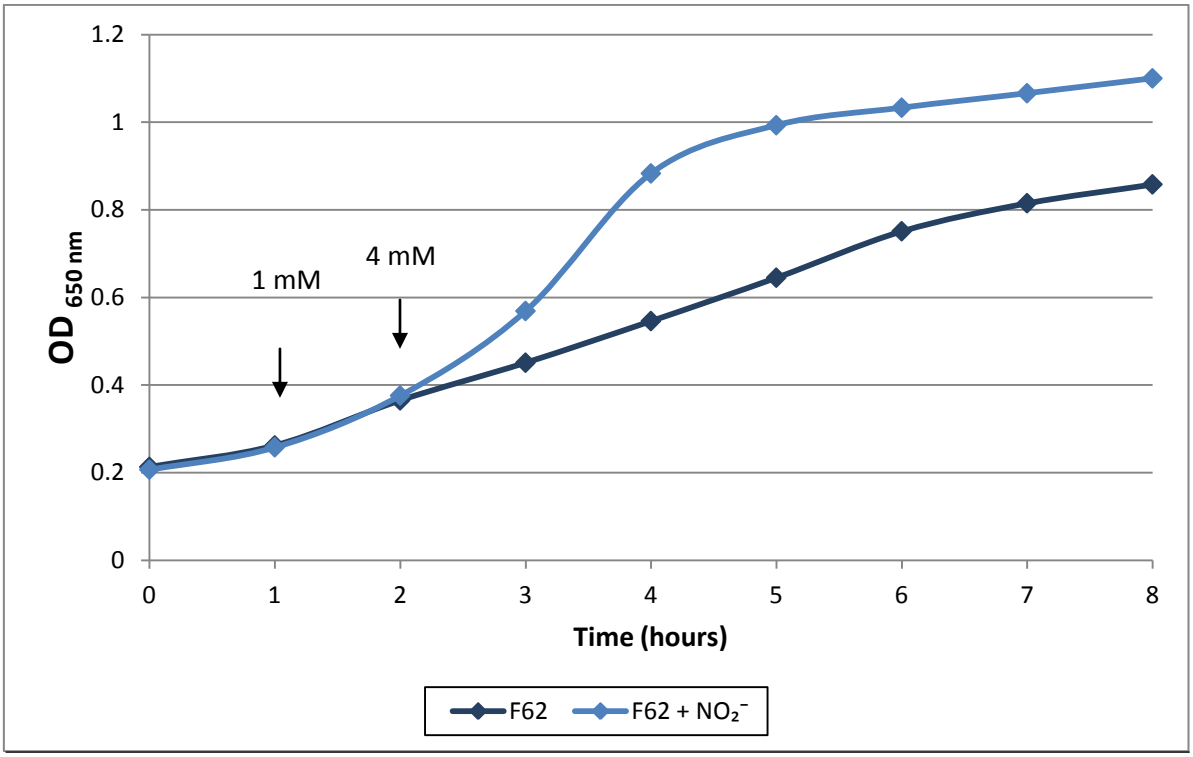


Figure 4.6 – Oxygen-limited growth of strain F62 supplemented with or without sodium nitrite

Arrows indicate the supplementation of cultures with sodium nitrite. Samples were taken at hourly intervals. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

possible that cytochrome c_2 is involved in electron donation to the second preferred terminal electron acceptor: nitrite. Both the parental strain F62 and strain JCGC851, which lacks the cytochrome c_2 coding sequence, were grown under oxygen-limited conditions as described above, supplemented with or without nitrite. As shown in Figure 4.7, the deletion of cytochrome c_2 results in a slightly slower growth rate compared to the parent strain. Although the addition of sodium nitrite to the culture increases the growth rate of strain JCGC851, the cytochrome c_2 deletion mutant is still slightly defective in oxygen-limited growth in the presence of sodium nitrite compared to the parent strain (Figure 4.7). The decrease in nitrite concentration during growth of cultures of the F62 parental strain and the cytochrome c_2 deletion mutant were compared. After the second addition of nitrite, the cytochrome c_2 deletion mutant reduced nitrite at a slower rate compared to the parent strain, resulting in a lag in nitrite reduction by the mutant strain (Figure 4.7). After this lag phase, the mutant appeared to reduce nitrite at a similar rate to the parent strain. The ability of the gonococcus to reduce nitrite is therefore affected by the absence of cytochrome c_2. This suggests a role for cytochrome c_2 in electron transfer to the nitrite reductase, AniA. The rate of nitrite reduction by the cytochrome c_2 deletion mutant was 48% lower than that of the parent strain, implicating cytochrome c_2 as a major pathway of electron transfer to AniA (Figure 4.8A). The abundance of AniA was identical in strains F62 and JCGC851 as shown by Western analysis (Figure 4.8B), suggesting that the decrease in nitrite reduction rate between these strains is due to a decrease in electron transfer to AniA and not a lower abundance of AniA in cells lacking cytochrome c_2. Although the nitrite reduction rate of the cytochrome c_2 mutant was lower than that of the parent strain, a considerable nitrite reduction ability is retained by the cytochrome c_2 deletion strain. This

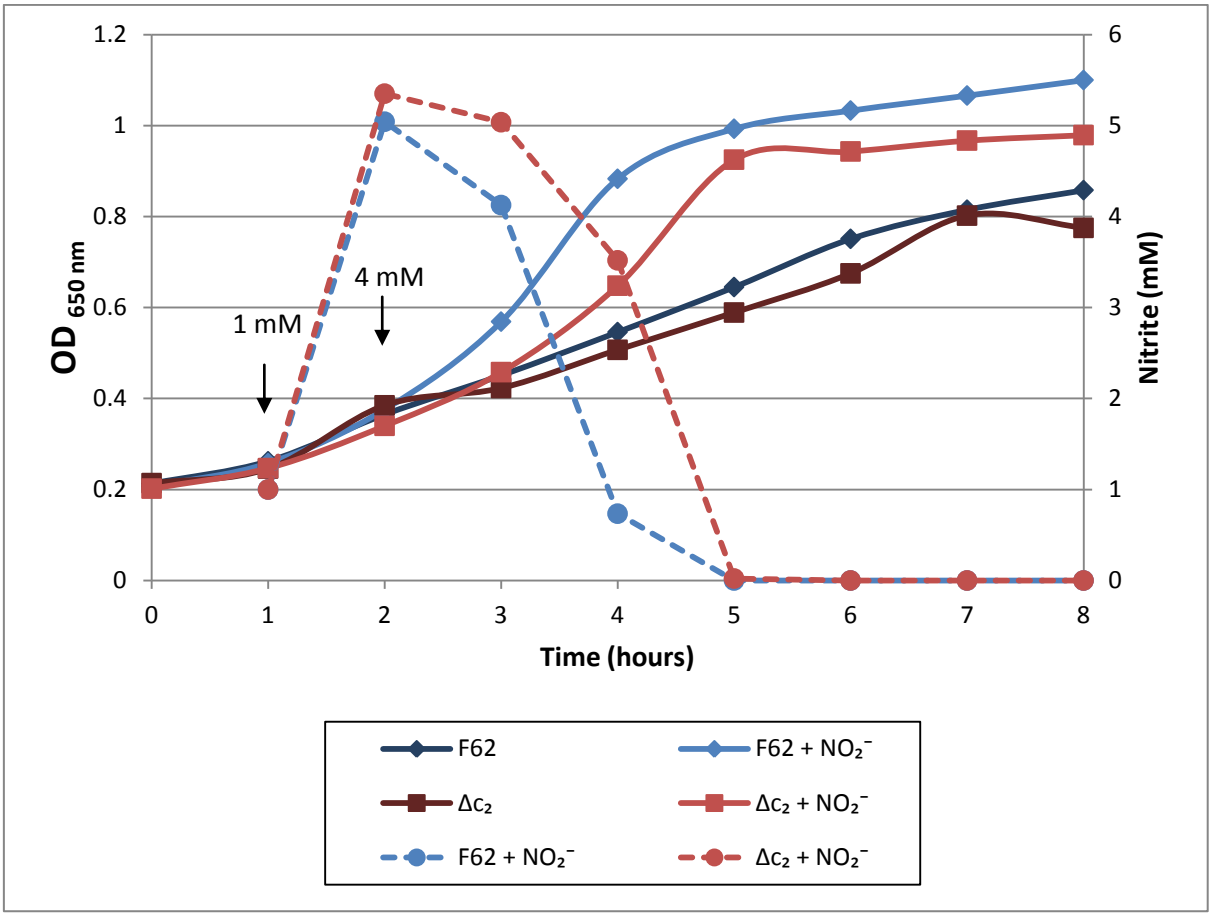


Figure 4.7 – Oxygen-limited growth and nitrite reduction during growth of strains F62 and JCGC851 (Δc_2), supplemented with or without nitrite

Solid lines indicate the optical density of cultures measured at a wavelength of 650 nm, and dotted lines indicate the concentration of nitrite present in the nitrite-supplemented cultures, which is plotted on the secondary y-axis. Samples were taken at hourly intervals. Arrows indicate the addition of sodium nitrite to relevant cultures. Growth curves shown are representative of duplicate cultures from at least two independent growth experiments.

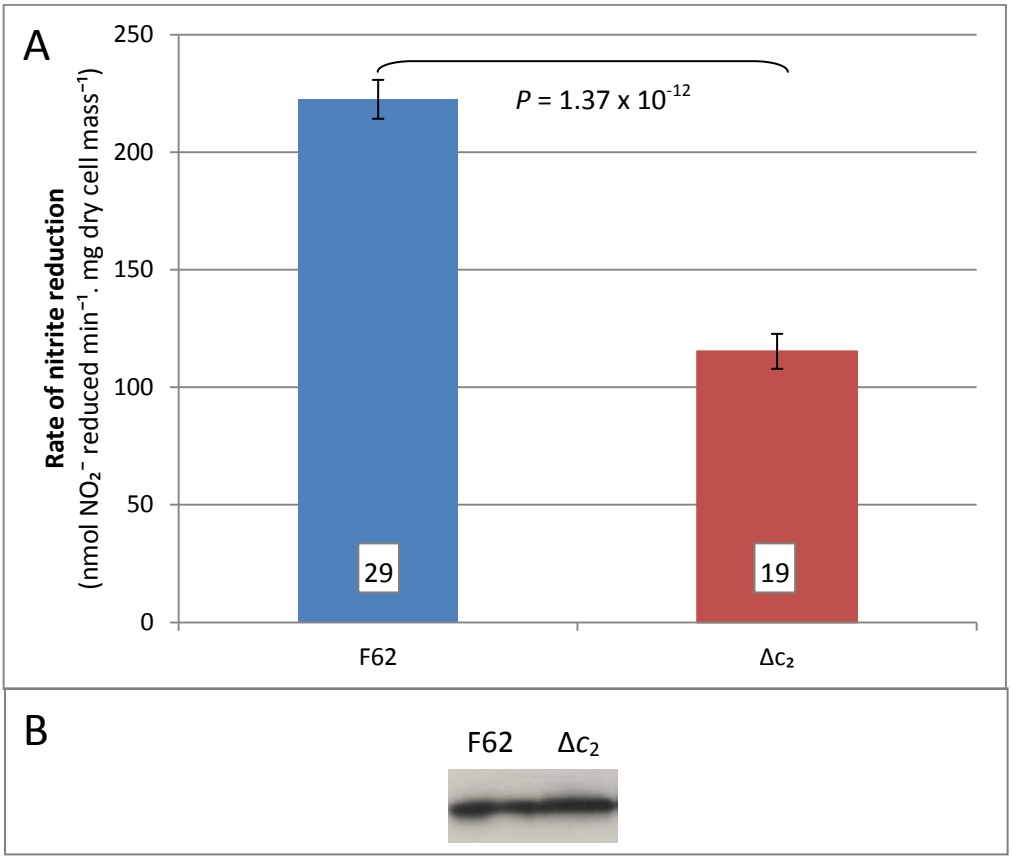


Figure 4.8 – Rates of nitrite reduction and accumulation of AniA protein in strains F62 and JCGC851 (Δ*c*$_2$)

A) Rates of nitrite reduction by whole, washed cells using lactate as an electron donor. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell mass^{-1} ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein (detected by Western blotting) during oxygen-limited growth. Samples were taken 5 hours after the commencement of growth as described above.

indicates the existence of multiple electron transfer pathways to AniA, the identities of which will be discussed in Chapter 5.

4.5 Overexpression of cytochrome c_2

Unlike the other 7 gonococcal *c*-type cytochromes, cytochrome c_2 cannot be visualised on an SDS-PAGE gel stained for the presence of covalently bound heme (Li *et al.*, 2010). This phenomenon was also observed for *Neisseria meningitidis* cytochrome c_x, a homologue of the gonococcal cytochrome c_2 (Deeudom *et al.*, 2008). There are a few possibilities to explain this discrepancy. It is known that cytochrome c_2 is transcribed in the gonococcus (Tovell, 2007); however, it was not known whether cytochrome c_2 mRNA was actually translated into protein. If cytochrome c_2 mRNA is translated, the lack of a visible cytochrome c_2 band on a heme-stained SDS-PAGE gel could be explained if heme was not attached to the protein. Another possibility is that cytochrome c_2 is expressed at very low levels such that the heme-staining procedure is not sensitive enough to visualise such a small amount of protein. To determine which hypothesis was correct, if any, it was decided to overexpress the cytochrome c_2 protein artificially. The Neisserial Insertional Complementation System (NICS) was used to insert an ectopically expressed copy of the *cccA* gene at an unlinked locus on the chromosome, the gene being under the control of a synthetic *lac* promoter. This construct was transformed into strain F62 to produce strain JCGC140. Strains F62 and JCGC140 were grown under oxygen-limited conditions supplemented with 5 mM sodium nitrite. In order to overexpress the ectopic copy of the *cccA* gene (encoding cytochrome c_2), 1 mM IPTG was added to relevant cultures immediately post-inoculation (Figure 4.9). On comparing the IPTG-induced culture of strain JCGC140 with the parental F62 strain, extra

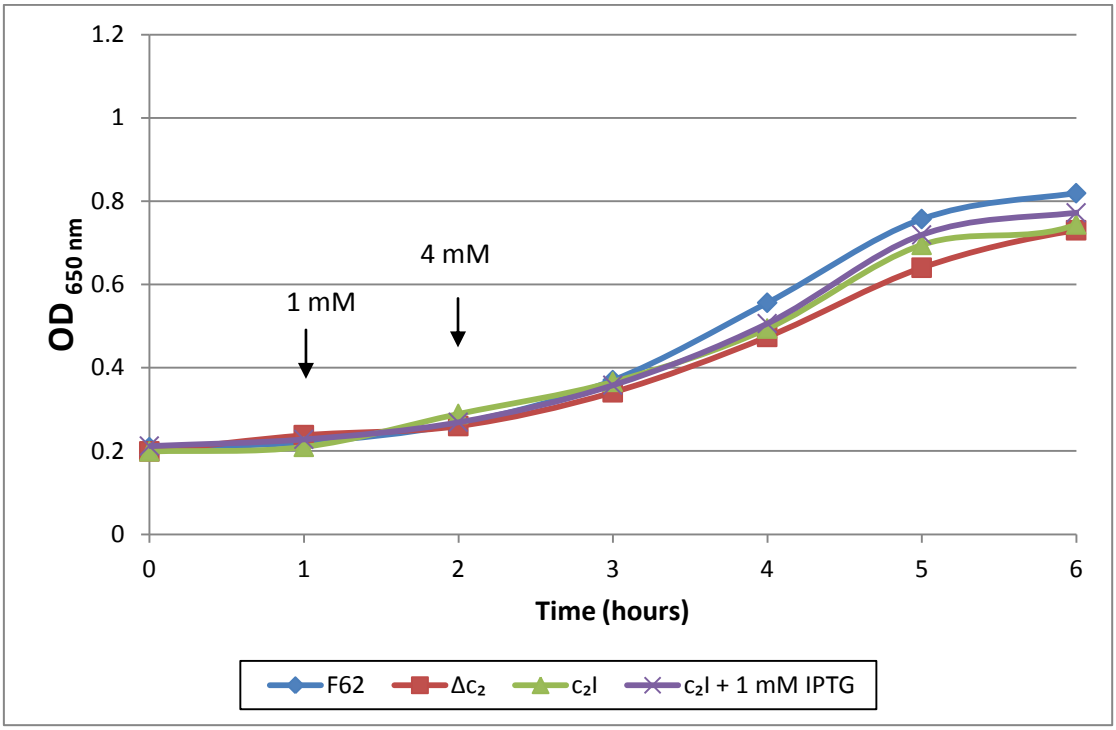


Figure 4.9 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc_2) and JCGC140 (c_2I)

Strain JCGC140 contains the additional copy of the *cccA* gene under the control of the *lac* promoter at the *lctP-aspC* locus. Cytochrome c_2 was ectopically expressed in relevant cultures by the addition of 1 mM IPTG at the start of the growth experiment. Arrows indicate the addition of sodium nitrite to cultures. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

heme-stained bands of protein can be seen on SDS-PAGE gels stained for the presence of covalently bound heme (Figure 4.10). Both bands represent the cytochrome c_2 protein, but the larger band is probably protein that has retained its *N*-terminal leader sequence and has not been released from the cytoplasmic membrane. This implies that the cytochrome has indeed been overexpressed, and that perhaps the membrane is saturated for fully post-translationally modified cytochrome c_2. This result indicates that the absence of the cytochrome c_2 band on SDS-PAGE gels stained for covalently bound heme is not due to the gonococcus being unable to translate the *cccA* mRNA into protein. It also shows that the gonococcus is able to incorporate the covalently bound heme group into the cytochrome c_2 protein, but is unable to cleave the *N*-terminal leader peptide from a population of the protein. One could speculate that cytochrome c_2 is only needed in very small quantities by the gonococcus, and that the heme-staining procedure is not sufficiently sensitive to detect the low levels of the protein. This is unexpected, as deletion of the *cccA* gene resulted in the loss of about half of the nitrite reduction ability of the gonococcus, implying that cytochrome c_2 is a very important component of the electron transfer chain to nitrite.

In the previous chapter, it was shown that the NICS could be used to complement mutant strains lacking either cytochromes c_4 or c_5. It was therefore assumed that this system would also allow the complementation of the nitrite reduction defect of the cytochrome c_2 deletion mutant. In order to complement the cytochrome c_2 deletion mutant, strain JCGC861 (lacking the cytochrome c_2 coding region replaced with a chloramphenicol resistance cassette) was transformed with the NICS containing cytochrome c_2 under the control of the *lac* promoter, producing strain JCGC142. Strains F62, JCGC851 (lacking the cytochrome c_2 coding region

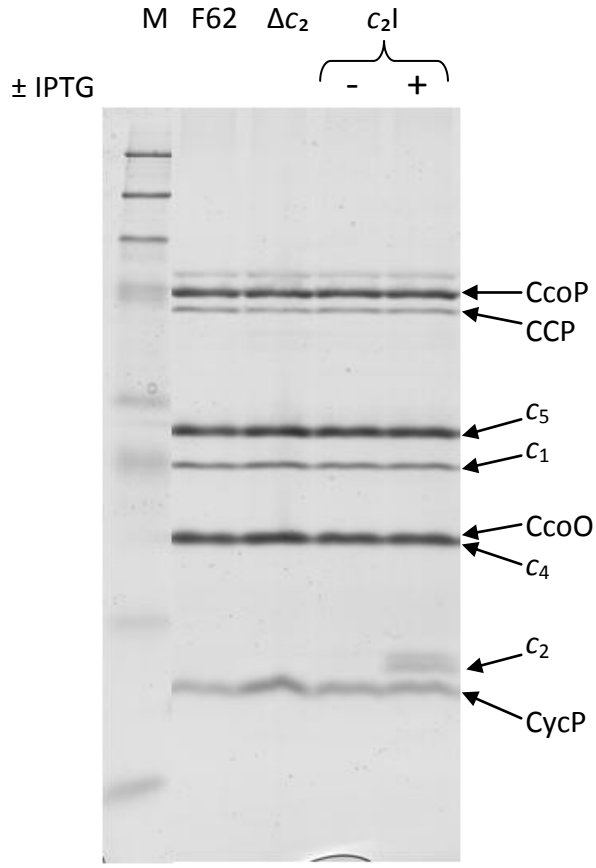


Figure 4.10 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc_2) and JCGC140 (c_2I)

Cultures were grown under oxygen-limited, nitrite-supplemented conditions. After 7 hours of growth, samples of bacteria were harvested, lysed and the proteins separated by SDS-PAGE and stained for covalently bound heme. The first lane contains protein marker; the second lane contains a sample from a culture of strain F62; the third lane is a sample from a culture of JCGC851; and the fourth and fifth lanes are from cultures of strain JCGC140 supplemented without and with 1 mM IPTG, respectively. Note that the cytochrome c_2 band, although faint, is a doublet.

replaced with a kanamycin resistance cassette) and JCGC142 (cytochrome c_2 deletion mutant complemented with the ectopically expressed copy of cytochrome c_2) were grown under oxygen-limited conditions in the presence or absence of 5 mM nitrite. Strain JCGC142 was grown in the presence and absence of 1 mM IPTG, which induces the expression of the ectopic copy of cytochrome c_2. The growth rate of strain JCGC142 is slightly improved on addition of IPTG, suggesting that the IPTG inducible copy of cytochrome c_2 is able to complement the loss of cytochrome c_2 to a degree (Figure 4.11A). However, the growth phenotype is not fully restored to the same level as the parent strain, even though the overexpression of cytochrome c_2 during these experiments was confirmed (Figure 4.12). In addition, strain JCGC142 is not able to reduce nitrite at the same rate as the parent strain (Figure 4.11B). To measure this quantitatively, the rates of nitrite reduction by strains F62, JCGC851 and JCGC142 were measured. The rate of nitrite reduction of JCGC142 cultures grown in the absence of IPTG was 54% that of the F62 parent strain, which was similar to the rate of the JCGC851 strain (Figure 4.13A). However, the addition of IPTG did not increase the rate of nitrite reduction of strain JCGC142, and therefore did not fully restore the parental rate of nitrite reduction. Western analysis was used to verify that this was not due to a lower accumulation of the AniA protein. The relative abundance of AniA was the same in all samples, suggesting that the lack of complementation was not due to a lower accumulation of AniA (Figure 4.13B). The fact that strain JCGC142 is able to overexpress holo-cytochrome c_2 (as shown in Figure 4.12) makes this result unexpected, as it seems that the cytochrome c_2 synthesised is unable to fully reduce the nitrite reductase, AniA. This phenomenon was not observed for the IPTG-inducible copies of cytochromes c_4 and c_5, which were fully able to complement the c_4 and c_5 deletion mutants, respectively, as shown in Chapter 3. This

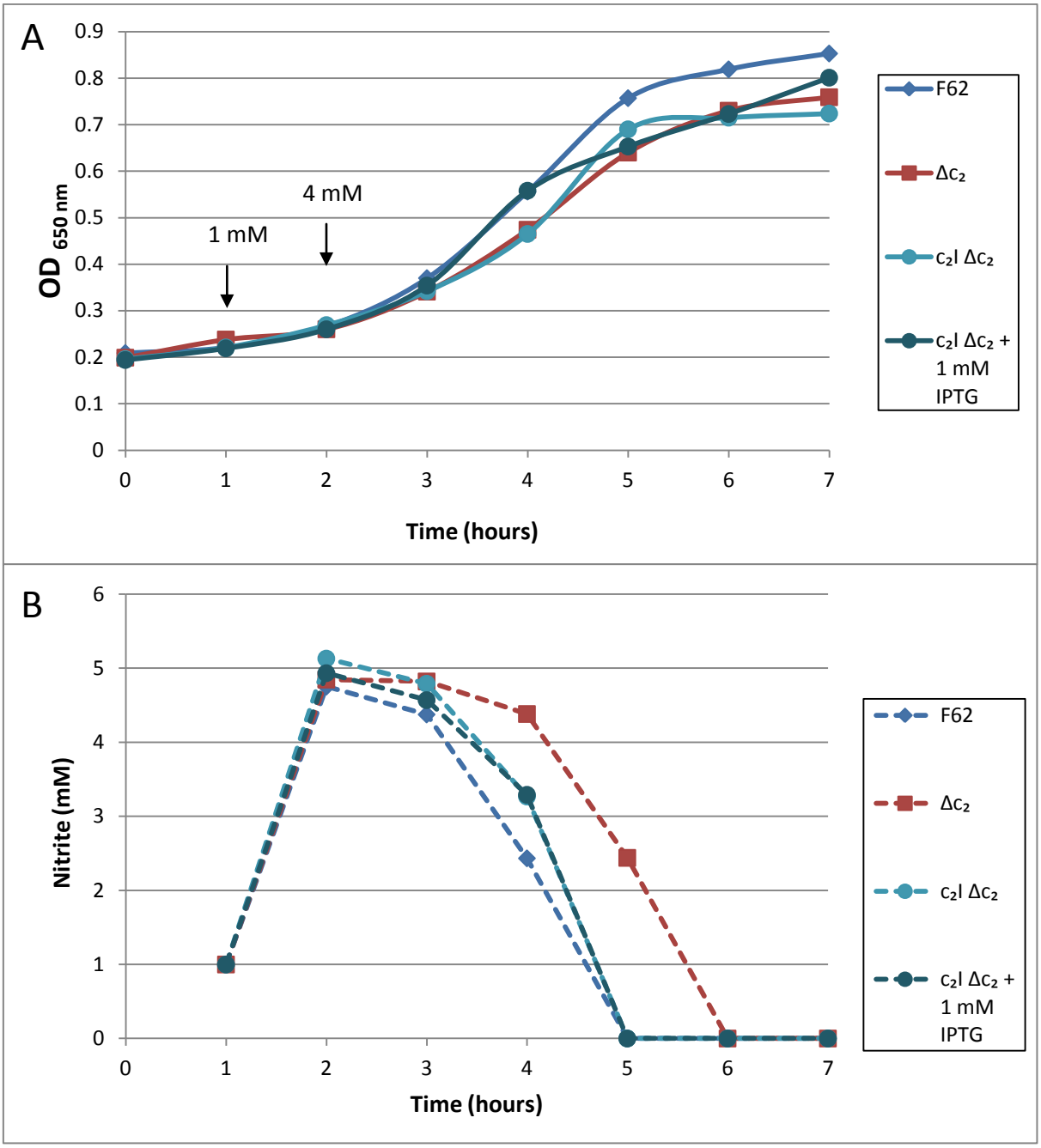


Figure 4.11 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc_2) and JCGC142 (c_2I Δc_2)

A) Growth of strains F62, JCGC851 and JCGC142. Arrows indicate the addition of sodium nitrite. Strain JCGC142 was grown in the absence and presence of 1 mM IPTG, in order to prevent or induce the expression of the ectopic copy of the *cccA* gene, respectively. Growth curves shown are representative of duplicate cultures from at least two independent growth experiments.

B) Concentration of nitrite in the growth media of cultures from Figure 4.11A.

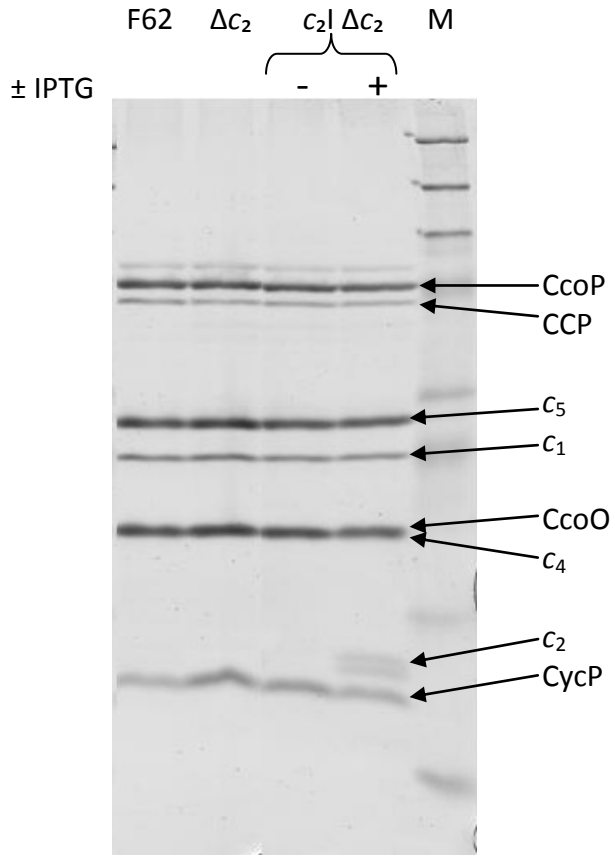


Figure 4.12 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc_2) and JCGC142 (c_2I Δc_2)

Cultures were grown under oxygen-limited, nitrite-supplemented conditions in the presence or absence of IPTG. After 7 hours of growth, samples of bacteria were harvested, lysed and the proteins separated by SDS-PAGE and stained for covalently bound heme. The first lane contains a sample from a culture of strain F62; the second lane is a sample from a culture of JCGC851; the third and fourth lanes are from cultures of strain JCGC142 supplemented without and with 1 mM IPTG, respectively; the fifth lane is protein marker.

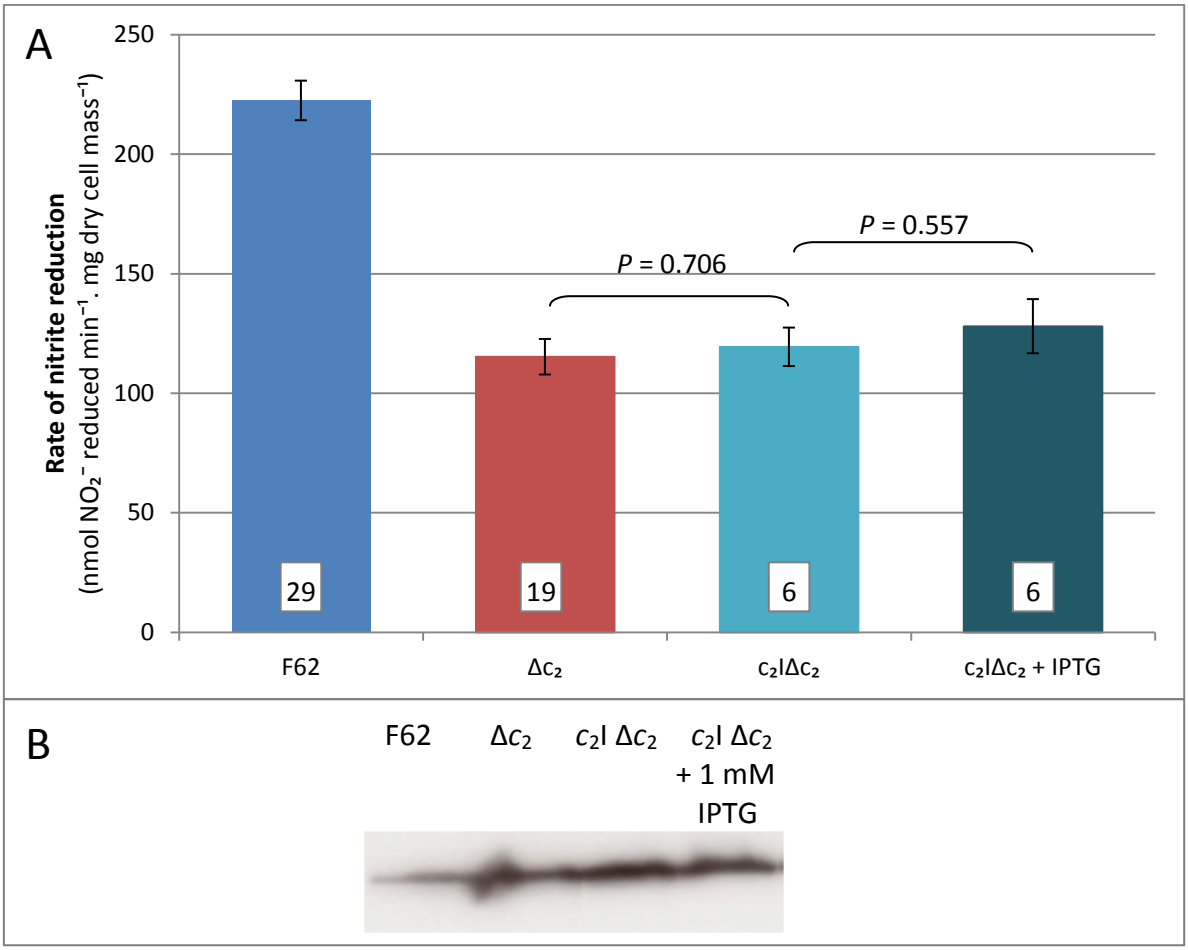


Figure 4.13 – Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC851 (Δc_2) and JCGC142 (c_2I Δc_2)

A) Rates of nitrite reduction by whole, washed cells using lactate as an electron donor. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. JCGC142 was grown in the absence and presence of 1 mM IPTG to induce the ectopic expression of cytochrome c_2 from the *lctP-aspC* locus. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein in strains F62, JCGC851, JCGC142 in the absence of IPTG and JCGC142 in the presence of 1 mM IPTG detected by Western blotting. Samples were taken 5 hours after the commencement of growth as described above.

suggests that an excess of cytochrome c_2 is in some way toxic to the cell, or perhaps that an abundance of cytochrome c_2 is detrimental to the functioning of AniA, either in being unable to receive electrons as efficiently from the respiratory chain or perhaps being unable to turnover its substrate as effectively.

4.6 Detection of cytochrome c_2 synthesis

In order to study the regulation of cytochrome c_2 synthesis in the gonococcus, a 3xFLAG tag was cloned onto the *C*-terminus of the cytochrome c_2 protein to create strain JCGC807. This would allow detection of both cytochrome c_2 apo-protein and holo-protein by Western analysis. As it was believed that cytochrome c_2 was expressed at a very low level compared to other gonococcal c-type cytochromes, the accumulation of 3xFLAG-tagged cytochrome c_2 was compared to the level of accumulation of 3xFLAG-tagged cytochrome c_4. Strains JCGC807, containing the 3xFLAG-tagged c_2 protein, and JCGC805, containing the 3xFLAG-tagged cytochrome c_4 protein, were grown under oxygen-limited conditions supplemented with 5 mM nitrite (Figure 4.14A). The growth rate of strain JCGC805 was much slower than that of the parental strain, even though the only difference between these strains was the presence of a 3xFLAG tag at the *C*-terminus of the cytochrome c_4 protein. The 3xFLAG tag contains many aspartic acid residues, which are negatively charged. It is possible that this negative charge affects the docking of cytochrome c_4 with the *cbb*$_3$ oxidase, thus decreasing the efficiency of electron transfer to oxygen and hence the growth rate of strain JCGC805. After 7 hours of growth, 1 mL samples of all four cultures were collected, pelleted, resuspended in sample buffer and lysed. Differing volumes of the 3xFLAG-tagged cytochrome c_2 sample were loaded onto an SDS-PAGE gel adjacent to the 3xFLAG-tagged

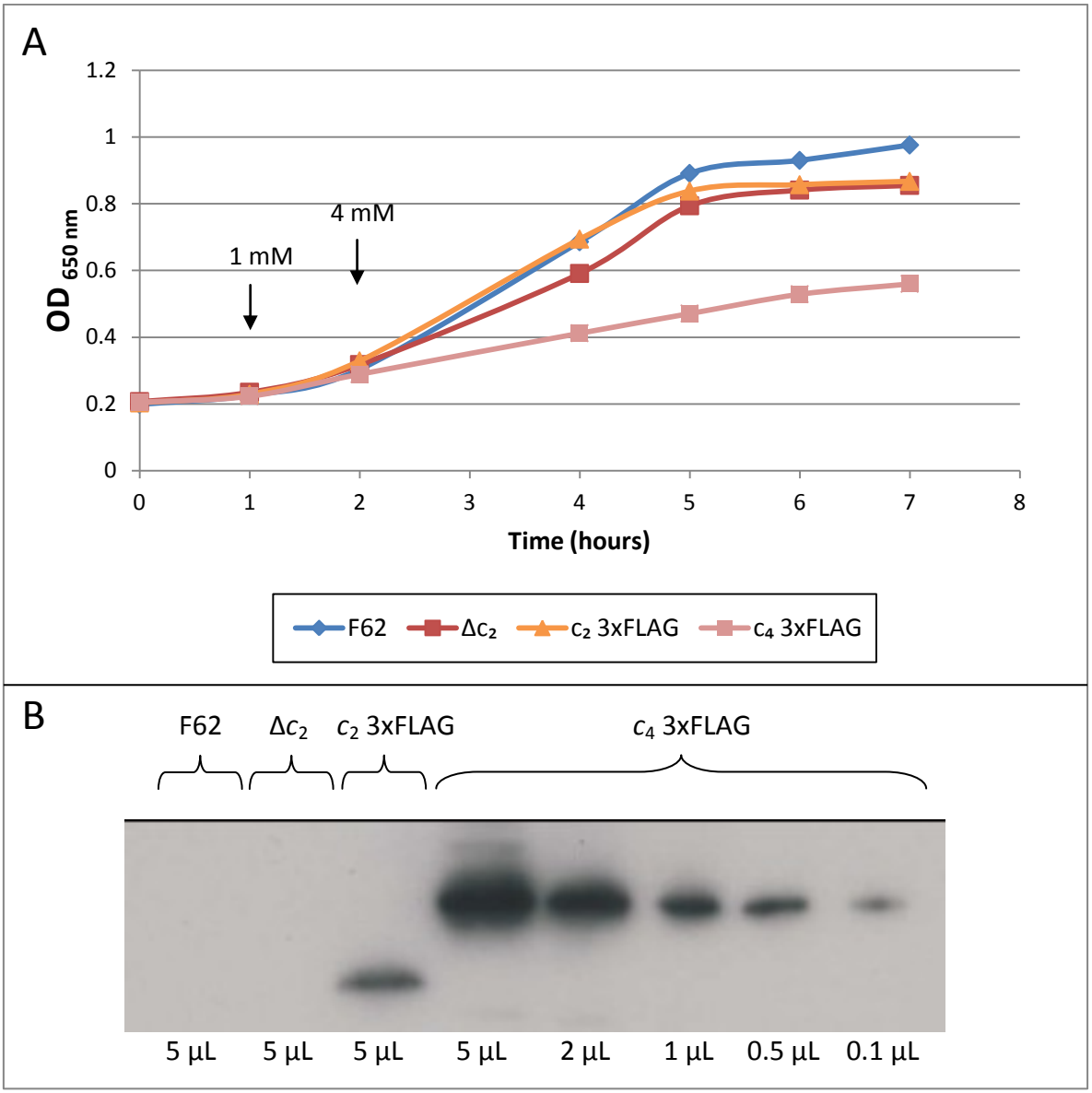


Figure 4.14 – Oxygen-limited nitrite-supplemented growth and accumulation of 3xFLAG-tagged proteins in cultures of strains F62, JCGC851 (Δc_2), JCGC807 (c_2^{3XFLAG}) and JCGC805 (c_4^{3XFLAG})

A) Oxygen-limited nitrite-supplemented growth. JCGC807 encodes a 3xFLAG-tagged copy of cytochrome c_2, and JCGC805 encodes a 3xFLAG-tagged copy of cytochrome c_4, both under the control of their natural promoters. Arrows indicate the addition of sodium nitrite. Growth curves shown are representative of two biological replicates grown on different days.

B) Accumulation of 3xFLAG-tagged proteins detected by Western blotting. In each lane 5 µL of sample was loaded, but for strain JCGC805 differing volumes were loaded (as shown above) in order to titrate in the relative abundance of the 3xFLAG-tagged cytochrome c_4 control strain. Samples were taken 5 hours after the commencement of growth.

cytochrome c_4 sample in order to determine the relative concentration of cytochrome c_2 compared to cytochrome c_4. Proteins within these samples were separated by SDS-PAGE, and the accumulation of FLAG-tagged proteins was then determined by Western analysis (Figure 4.14B). On comparing the relative intensity of FLAG-tagged protein, the intensity of lane 3 (where 5 µl of culture JCGC807 was loaded) corresponds roughly to lane 7, where 0.5 µl of culture JCGC805 was loaded. This implies that the level of cytochrome c_2 in gonococcal cells is approximately 10-fold lower than that of cytochrome c_4. The absence of a band corresponding to cytochrome c_2 on an SDS-PAGE gel stained for covalently bound heme can therefore be explained by the limited sensitivity of the heme staining procedure and not the inability of heme to be incorporated into the cytochrome c_2 apo-protein (as shown by the ability to overexpress cytochrome c_2 as described earlier). This reinforces the hypothesis that cytochrome c_2 is expressed at a very low level compared to the other electron transfer chain components.

4.7 Regulation of cytochrome c_2 expression

As cytochrome c_2 appeared to be an electron donor to the nitrite reductase, AniA, it was possible that its expression might be induced, like AniA, by the presence of nitrite (Householder *et al.*, 1999; Overton *et al.*, 2006). Strains F62, JCGC851 and JCGC807 were grown under oxygen-limited conditions supplemented with 5 mM sodium nitrite. Strain JCGC807 was also grown under oxygen-limited conditions without nitrite, in order to determine whether nitrite would induce expression of 3xFLAG-tagged cytochrome c_2 (Figure 4.15A). After 1 hour, 2 hours, 4 hours and 6 hours of growth, 1 mL samples were taken from each culture and the presence of 3xFLAG-tagged cytochrome c_2 was detected using Western

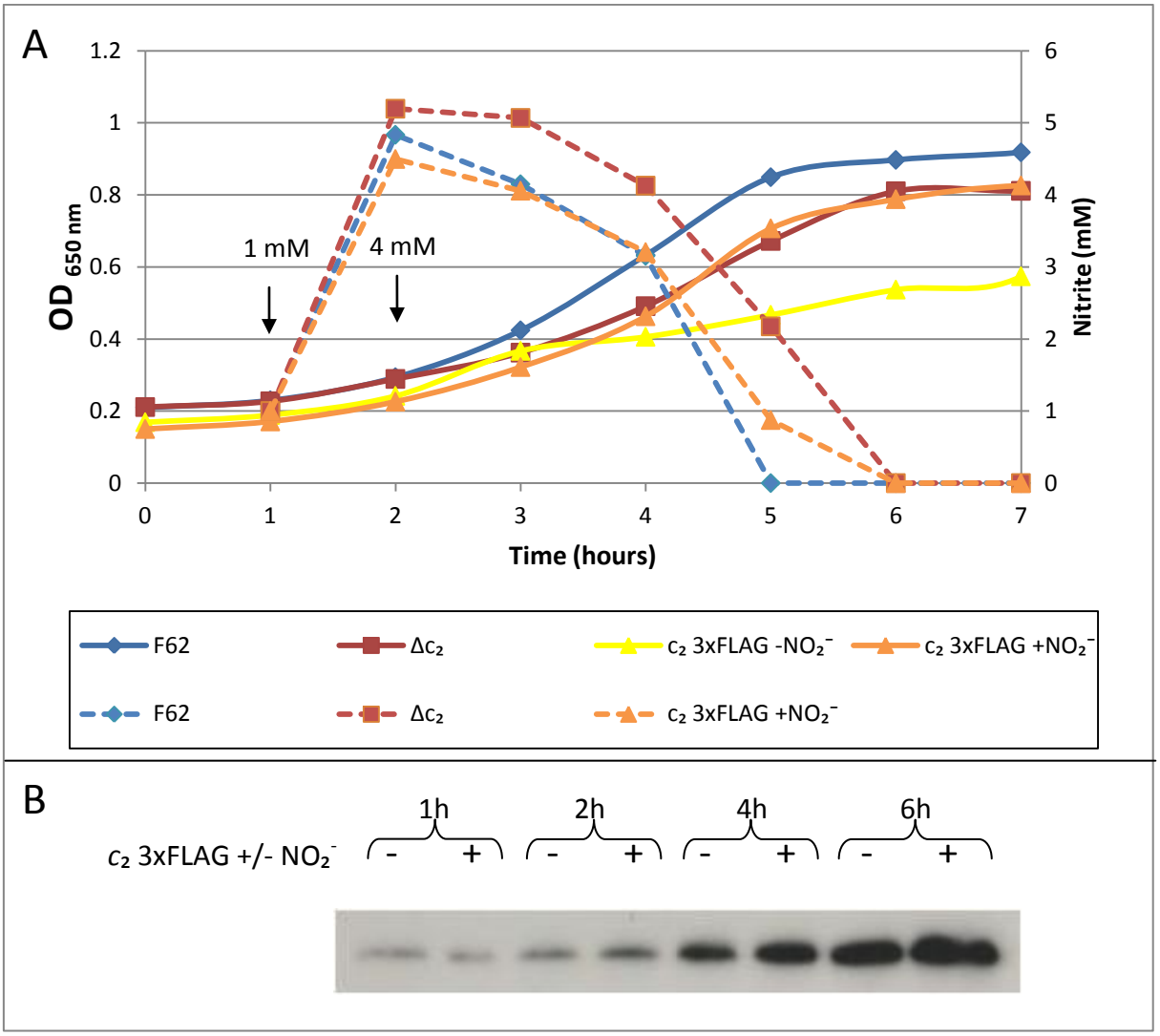


Figure 4.15 – Oxygen-limited growth, nitrite reduction during growth, and accumulation of 3xFLAG-tagged cytochrome c_2 protein in strains F62, JCGC851 (Δc_2) and JCGC807 (c_2^{3XFLAG})

A) Oxygen-limited growth and nitrite reduction during growth. Arrows indicate the addition of sodium nitrite. Strain JCGC807 was also grown in the absence of nitrite. Solid lines indicate the optical density of cultures measured at a wavelength of 650 nm, and dotted lines indicate the concentration of nitrite present in the nitrite-supplemented cultures, which is plotted on the secondary y-axis. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Accumulation of 3xFLAG-tagged cytochrome c_2 protein in strain JCGC807 (encoding a 3xFLAG-tagged copy of cytochrome c_2 under the control of its natural promoter) detected by Western blotting. Samples were taken after 1 hour, 2 hours, 4 hours and 6 hours of growth from cultures in the absence and presence of nitrite, designated by the – and + signs, respectively.

analysis. FLAG-tagged cytochrome c_2 accumulated during growth in both the presence and absence of nitrite; however, it appeared that cultures supplemented with nitrite contained a higher concentration of cytochrome c_2 protein compared with unsupplemented cultures (Figure 4.15B). As all samples loaded on the SDS-PAGE gel were normalised to the optical density of the culture, this difference in intensity could be perceived as an increase in expression of FLAG-tagged cytochrome c_2.

The way in which cytochrome c_2 expression is regulated is unknown and there do not appear to be any obvious transcription factor binding sites upstream of the *cccA* gene. However, on submitting the upstream sequence to the MFold program (Zuker, 1989), a region of potential stem loop formation was noted (Figure 4.16). Gene expression can be regulated by stem loop formation, which can either promote or hinder the transcription of a gene or the translation of the resulting message (Bevilacqua & Blose, 2008). The potential stem loop structure that could form upstream of the *cccA* coding sequence could occlude the Shine-Dalgarno ribosomal binding site, and could therefore potentially affect the translation of the *cccA* transcript into protein. To determine whether this phenomenon was physiologically relevant, site-directed mutagenesis was used to disrupt potential stem loop formation at that locus. Bases located 22 bp, 21 bp and 20 bp upstream of the ATG start codon of the *cccA* gene were mutated from T to A, T to A and C to G, respectively. This would prevent this region of the mRNA molecule from pairing with part of the Shine-Dalgarno site. To determine whether mutating the potential stem loop would affect the ectopic expression of cytochrome c_2, the constructs containing both the unmutated and mutated form of this potential stem loop were transformed into strain JCGC851, i.e. a background where the only

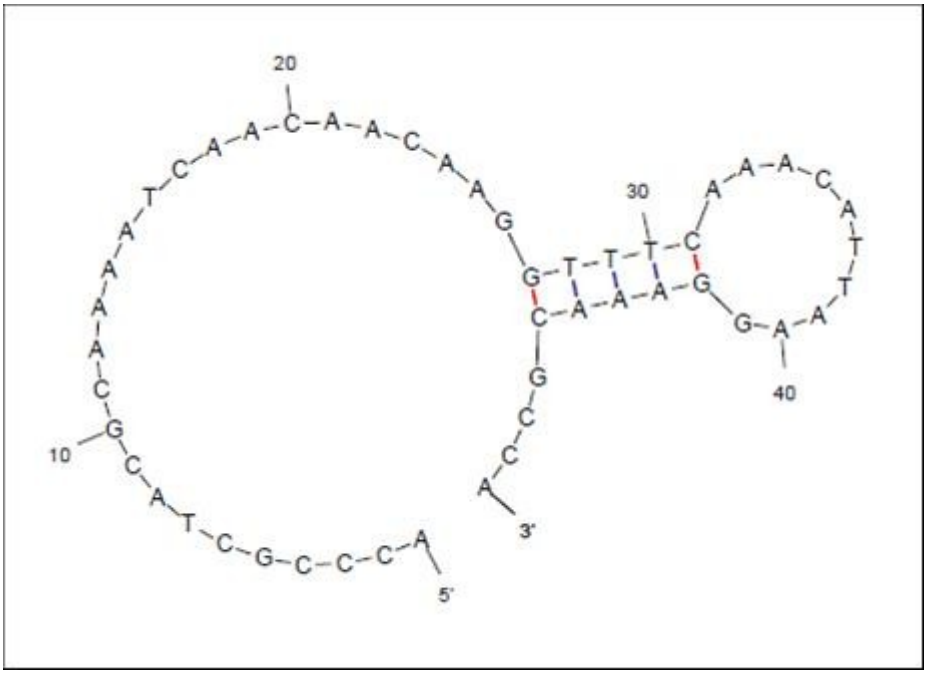


Figure 4.16 – Diagram showing the potential stem loop structure that could form upstream of the translation start site of the *cccA* gene

The Shine-Dalgarno sequence is labelled as numbers 40-43, and the bases that were mutated by site-directed mutagenesis are labelled as numbers 29 (T converted to A), 30 (T converted to A) and 31 (C converted to G). These are referred to as mutations T-22A, T-21A and C-20G as described in the text, where the - sign denotes the number of base pairs upstream of the ATG start codon. Modified from the MFold program (Zuker, 1989).

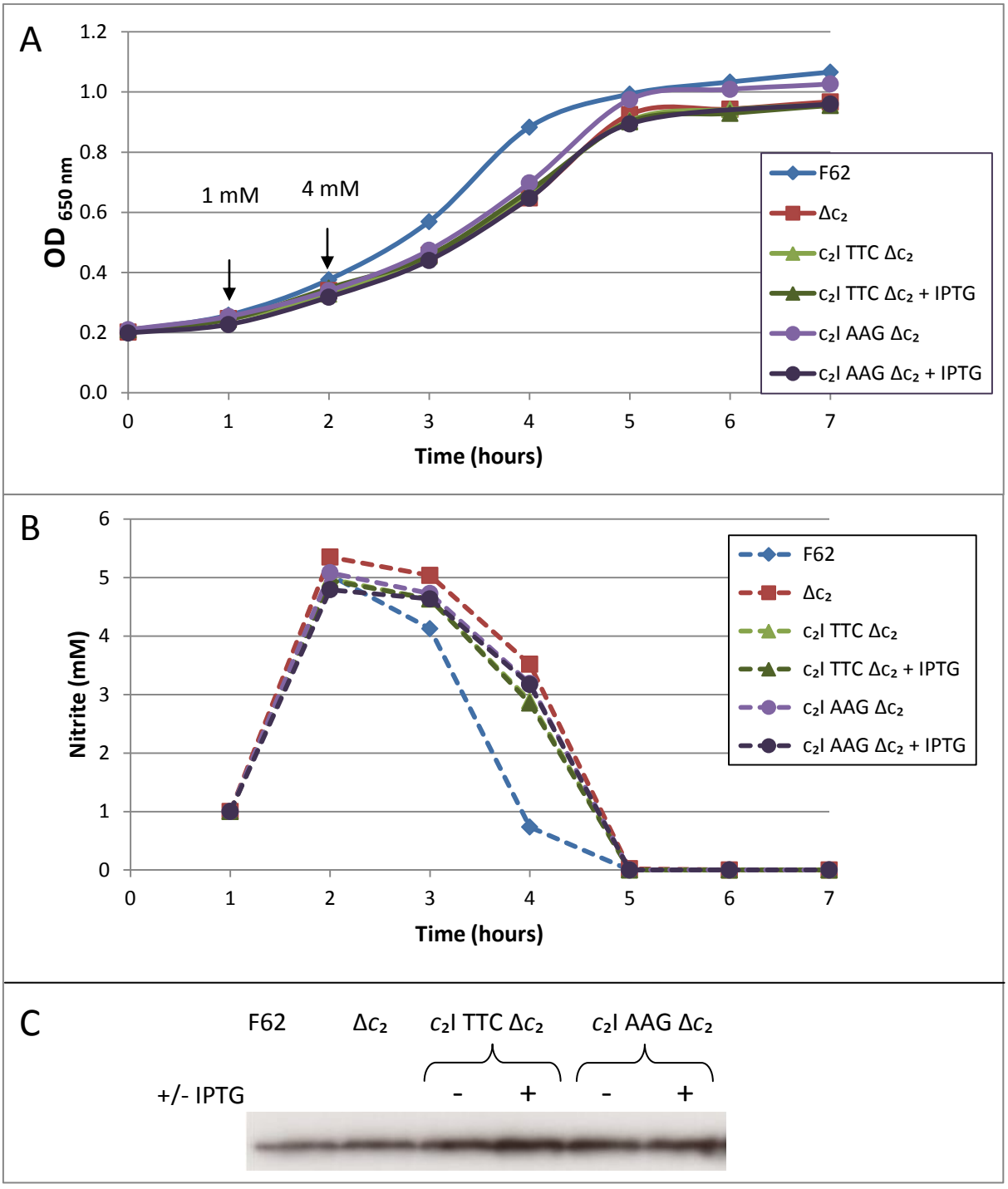
copy of cytochrome c_2 is at the *lctP-aspC* locus and is under the control of the *lac* promoter. The resulting strains JCGC152 and JCGC153 therefore both lacked cytochrome c_2 at its natural locus but contained the IPTG-inducible copy of *cccA* with the potential stem loop unmutated or mutated, respectively. Strains F62, JCGC851 (Δc₂), JCGC152 (c_2I TTC Δc_2) and JCGC153 (c_2I AAG Δc_2) were grown under oxygen-limited conditions supplemented with 5 mM sodium nitrite, and 1 mM IPTG was added to relevant cultures immediately post-inoculation. The rate of nitrite reduction during growth by strains JCGC152 and JCGC153 was similar to that of the cytochrome c_2 deletion mutant (strain JCGC851), and the abundance of AniA was the same in all cultures (Figures 4.17A and 4.17B). This suggests that, as described previously, the ectopic expression of cytochrome c_2 cannot complement for the loss of cytochrome c_2 from its natural location on the chromosome. The amount of cytochrome c_2 expressed by strains JCGC152 and JCGC153 was comparable (Figure 4.18), implying that the potential stem loop structure found has no apparent effect on cytochrome c_2 expression. However, this does not prove that no such regulation occurs at the natural cytochrome c_2 promoter.

4.8 Discussion

In the previous chapter it has been shown that cytochromes c_4 and c_5 are electron donors to the sole terminal oxidase in the gonococcus. However, the only phenotype reported for cytochrome c_2 in this chapter was a slightly increased oxygen reduction rate in the absence of cytochrome c_2. It was therefore speculated that cytochrome c_2 could act as an electron donor to the alternative terminal electron acceptor, nitrite, via the nitrite reductase, AniA. The deletion of cytochrome c_2 resulted in a 48% decrease in nitrite reduction rate but had no

Figure 4.17 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc_2), JCGC152 (c_2I TTC Δc_2) and JCGC153 (c_2I AAG Δc_2)

A) Oxygen-limited, nitrite supplemented growth. Arrows indicate addition of sodium nitrite. Strains JCGC152 and JCGC153 were grown in the absence and presence of 1 mM IPTG. Growth curves shown are representative of duplicate cultures from two independent growth experiments.
B) Concentration of nitrite in the growth media of cultures from Figure 4.17A.
C) Accumulation of AniA protein. Samples were taken 5 hours after the commencement of growth.


A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 650 nm
1 mM
4 mM
0
1
2
3
4
5
6
7
Time (hours)
F62
Δc2
c2I TTC Δc2
c2I TTC Δc2 + IPTG
c2I AAG Δc2
c2I AAG Δc2 + IPTG
B
6
5
4
3
2
1
0
Nitrite (mM)
0
1
2
3
4
5
6
7
Time (hours)
F62
Δc2
c2I TTC Δc2
c2I TTC Δc2 + IPTG
c2I AAG Δc2
c2I AAG Δc2 + IPTG
C
F62
Δc2
c2I TTC Δc2
c2I AAG Δc2
+/- IPTG
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+
-
+

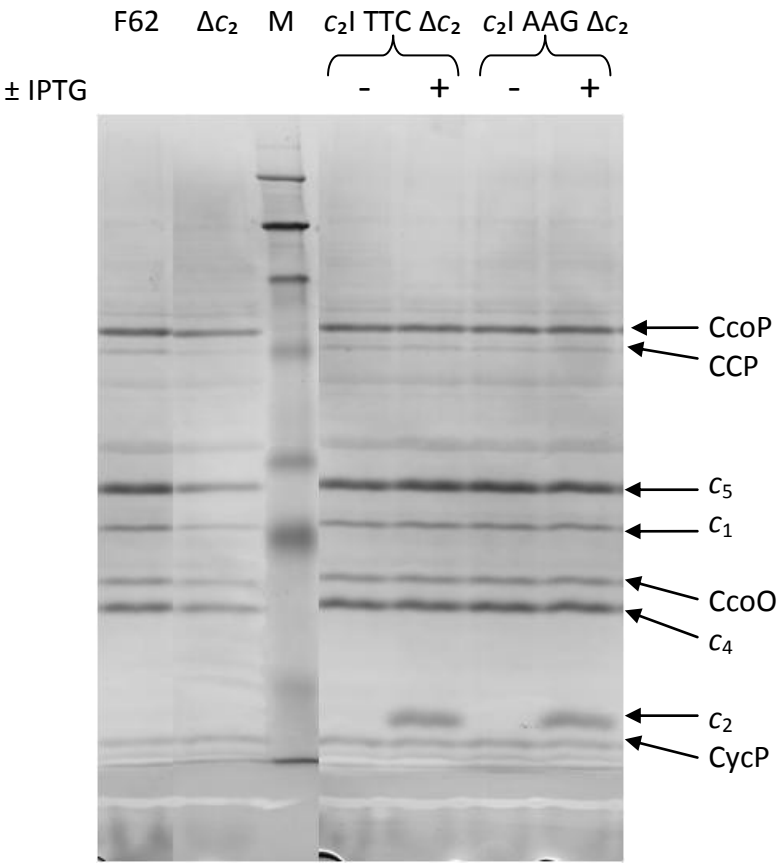


Figure 4.18 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc_2), JCGC152 (c_2I TTC Δc_2) and JCGC153 (c_2I AAG Δ c_2)

Cultures were grown under oxygen-limited, nitrite supplemented conditions in the presence or absence of IPTG. Samples were taken after 5 hours of growth. The first lane contains a sample from a culture of strain F62; the second lane is a sample from a culture of JCGC851; the third lane is protein marker; the fourth and fifth lanes are from cultures of strain JCGC152 supplemented without and with 1 mM IPTG, respectively; and the sixth and seventh lanes are from cultures of stain JCGC153 supplemented without and with 1 mM IPTG, respectively.

effect on AniA synthesis compared to the parent strain, suggesting a role for cytochrome c_2 in electron transfer to nitrite.

4.8.1 The low abundance of cytochrome c_2

All but one of the gonococcal *c*-type cytochromes can be visualised on an SDS-PAGE gel stained for the presence of covalently bound heme, the exception being cytochrome c_2. This suggested that either heme does not bind to the CXXCH motif in cytochrome c_2, or that the abundance of cytochrome c_2 is too low to be detected by the heme-staining procedure. Two lines of enquiry disproved the former hypothesis. Firstly, overexpression of cytochrome c_2 allowed the visualisation of mature holo-cytochrome c_2 on a heme-stained polyacrylamide gel. Secondly, the relative abundance of 3xFLAG-tagged cytochrome c_2 was found to be 10-fold lower than a 3xFLAG-tagged cytochrome c_4 control. This supports the hypothesis that the heme-staining procedure is insufficiently sensitive to visualise the low abundance of cytochrome c_2, unless it is overexpressed.

One possible explanation for the low abundance of cytochrome c_2 could be that it is an unstable, easily degraded protein. This could be tested by using a pulse-chase technique. Cells could be pulsed with heavy nitrogen (^{15}N) then flooded with ^{14}N, and the relative abundance of each isotope measured by mass spectroscopy (Green *et al.*, 1982). Alternatively, cells could be pulsed with radioactive sulphur (^{35}S) then flooded with ^{32}S, and the level of radioactive cytochrome c_2 could be compared to the level of radioactivity of the other *c*-type cytochromes (Govezensky *et al.*, 1991). If cytochrome c_2 is an unstable protein, the relative level of ^{15}N or ^{35}S cytochrome c_2 would be lower than the other *c*-type

cytochromes, as the cytochrome c_2 would have been degraded.

4.8.2 The expression of cytochrome c_2 does not appear to be induced by nitrite

It has been reported that the relative amount of gonococcal *c*-type cytochromes found per cell increases during growth due to the decrease in oxygen concentration in cultures. It is believed that the presence of oxygen can inhibit heme attachment to *c*-type cytochromes. Therefore, as the cell density increases in a given culture, the oxygen concentration decreases, lowering the inhibitory effect of oxygen on heme attachment and therefore increasing the abundance of *c*-type cytochromes (Tovell, 2007). As mentioned previously, nitrite enhances oxygen-limited growth of the gonococcus, resulting in an increased growth rate compared to unsupplemented cultures. This means that nitrite-supplemented cultures attain a higher cell density more rapidly than unsupplemented cultures, and therefore the abundance of all *c*-type cytochromes at a given time point will be higher in nitrite-supplemented cultures than in unsupplemented cultures. It is therefore likely that the higher abundance of 3xFLAG-tagged cytochrome c_2 in cultures supplemented with nitrite compared to unsupplemented cultures was as a result of a greater overall accumulation of *c*-type cytochromes, and not due to the induction of cytochrome c_2 expression by nitrite. Furthermore, the fact that cytochrome c_2 still accumulates in the absence of nitrite suggests that cytochrome c_2 is expressed at a low constitutive level.

4.8.3 The overexpression of cytochrome c_2 cannot complement a cytochrome c_2 deletion mutant

Although the overexpression of cytochrome c_2 was successful, the system was unable to

complement the absence of the original copy of cytochrome c_2 in terms of growth defects and nitrite reduction rates. This was unexpected, as the same complementation system was able to complement the loss of cytochromes c_4 and c_5 by inducing expression of the relevant cytochromes with IPTG. This suggests that an excess of cytochrome c_2 in the bacterium is detrimental to the ability of the gonococcus to reduce nitrite. There are many possible reasons for this. One possibility is that the abundance of the soluble electron carrier disturbs the redox balance in the periplasm in some way. If the midpoint potential of cytochrome c_2 is particularly high, an increase in the concentration of cytochrome c_2 might oxidise the electron transfer pathway. The most similar, characterised *c*-type cytochrome to gonococcal cytochrome c_2 is cytochrome c_{552} from *Thermus thermophilus*, which has a midpoint potential of + 200 mV (Fee *et al.*, 2000). Assuming that the midpoint potential of these two cytochromes is similar, it is unlikely that cytochrome c_2 would be sufficiently oxidizing to affect the redox balance in the periplasm. An alternative possibility is that cytochrome c_2 in some way saturates AniA with electrons, or perhaps sterically hinders the functionality of the nitrite reductase. Either way, the evidence suggests that overexpressing the cytochrome c_2 protein does in fact impair the transfer of electrons to AniA in some way, or perhaps inhibits the enzymic conversion of nitrite to nitric oxide.

4.8.4 A novel role for cytochrome c_2?

Cytochrome c_2 is very important for the reduction of nitrite by the gonococcus. Therefore it would be expected that the stoichiometry of cytochrome c_2 to the other *c*-type cytochromes, such as cytochrome c_4, should be of the same order. It is therefore surprising that the abundance of cytochrome c_2 is very low. One possibility is that cytochrome c_2 is a

very unstable protein, and that it is degraded easily by proteolysis. The low constitutive level of expression of cytochrome c_2 would therefore provide a low enough abundance of cytochrome c_2 to present no toxicity problems, and would compensate for the instability of the protein, allowing sufficient electrons to reach AniA from the inner membrane. Nevertheless, this raises the following question: how can cytochrome c_2 play such a major role in electron transfer to AniA when its abundance is so low? One possibility is that cytochrome c_2 is not an electron donor, but perhaps a regulator or sensor of redox control or some other homeostatic mechanism. These hypotheses will be discussed further in Chapter 6.

Chapter 5

Electron donors to the nitrite reductase, AniA

5.1 Introduction

AniA, a member of the NirK family, is the only nitrite reductase present in *Neisseria gonorrhoeae*. It has two copper sites that function together to allow the one-electron reduction of nitrite to nitric oxide (Boulanger & Murphy, 2002). The electrons required for nitrite reduction are known to originate from redox proteins on the inner membrane, but these electron donors have not been identified. Unlike many other nitrite reductases, which are located in the cytoplasm, AniA is tethered to the outer membrane of the gonococcus. The precise orientation of AniA in the outer membrane is a point of controversy. One model suggests that AniA must project into the periplasm to allow AniA to make contact with other redox components of the respiration chain (Boulanger & Murphy, 2002). However, as antibodies directed towards AniA are often synthesised in the human host, this suggests that AniA is exposed to the external milieu (Clark *et al.*, 1988). Nevertheless, it is possible that these antigens could be released as a result of bacterial lysis, to which gonococcal cultures are susceptible (Morse & Bartenstein, 1974). It has also been suggested that the AniA protein could provide protection to the gonococcus against human serum (Cardinale & Clark, 2000). Whichever model is correct, the fact remains that the reduction of nitrite by AniA is a mechanism required by the gonococcus in order to conserve energy during oxygen-limited growth (Knapp & Clark, 1984; Rock *et al.*, 2005).

To date, the pathway of electron transfer from inner membrane redox proteins to AniA is unknown. However, electron transfer pathways to nitrite reductases in other bacteria have been characterised. *Paracoccus denitrificans* has a cd_1-type nitrite reductase, which receives electrons from two routes, namely cytochrome c_{550} and pseudoazurin (Pearson *et al.*, 2003).

Rhodobacter sphaeroides has a NirK-like nitrite reductase, similar to AniA, which receives electrons from CycA (designated cytochrome c_2 in *R. sphaeroides*), cytochrome c_Y and a further, unknown electron donor (Laratta *et al.*, 2006). *Bradyrhizobium japonicum* also encodes a copper-containing nitrite reductase, which receives electrons from only one electron donor, namely cytochrome c_{550} (Bueno *et al.*, 2008). Although electron donors to nitrite reductases vary between different bacteria, these examples offer insight into the possible identity of redox proteins involved in electron transfer to gonococcal AniA. This chapter will focus on the elucidation of electron transfer pathways to the gonococcal nitrite reductase, AniA.

Parts of this chapter have been published in Hopper *et al.*, 2009, the first author of which is the author of this work.

5.2 Cytochromes c_4 and c_5 as potential electron donors to AniA

In Chapter 4, it was reported that cytochrome c_2 might be an electron donor to AniA. As the deletion of cytochrome c_2 did not completely deplete the ability of the gonococcus to reduce nitrite, other pathways of electron transfer to AniA must exist. Consequently, one could hypothesise that cytochromes c_4 or c_5 could provide further pathways for electron transfer across the gonococcal periplasm to the nitrite reductase, AniA. As described previously in Chapter 3, both cytochromes c_4 and c_5 constitute pathways of electron transfer to oxygen. To determine whether either of these cytochromes were also involved in electron transfer to AniA, single mutants defective in either cytochromes c_4 or c_5 were assayed for their ability to reduce nitrite. Strains F62, JCGC800 (Δc_4) and JCGC850 (Δc_5) were grown under oxygen-

limited conditions supplemented with sodium nitrite. Bacteria were harvested immediately after all of the nitrite had been reduced by cultures to ensure that AniA expression had been induced. The rates of nitrite reduction by washed bacterial suspensions were measured using the whole cell nitrite reduction assay (as described in Chapter 2). The rate of nitrite reduction by a mutant lacking cytochrome c_5 was 41% lower than the parent strain, and a mutant lacking cytochrome c_4 had a 64% lower rate of nitrite reduction than the parent strain (Figure 5.1A). The amount of AniA protein present in each culture was identical, which shows that the decreased rate of nitrite reduction in mutants compared to the parent strain is due to decreased electron transfer to AniA (Figure 5.1B). This implies that both cytochromes c_5 and c_4 could be transferring electrons to AniA. However, further electron transfer pathways must remain as neither of these mutants was completely deficient in their ability to reduce nitrite.

5.3 The novel third heme group of cytochrome CcoP

Cytochrome *cbb*$_3$ oxidase from most bacteria includes multiple subunits, including two *c*-type cytochromes: CcoP and CcoO. The CcoP subunit is typically a di-heme *c*-type cytochrome that receives electrons from soluble *c*-type cytochromes located in the periplasm. Electrons are thought to be transferred from CcoP via CcoO to the copper-containing catalytic site for oxygen reduction located in CcoN (Buschmann *et al.*, 2010). Interestingly, the CcoP subunit of the gonococcal cytochrome *cbb*$_3$ oxidase is significantly larger than the orthologous subunits from other bacteria, and contains a *C*-terminal extension that might be sufficiently long to span the periplasm (Figure 5.2). In all *Neisseria* species except for *Neisseria meningitidis*, this *C*-terminal domain includes an additional

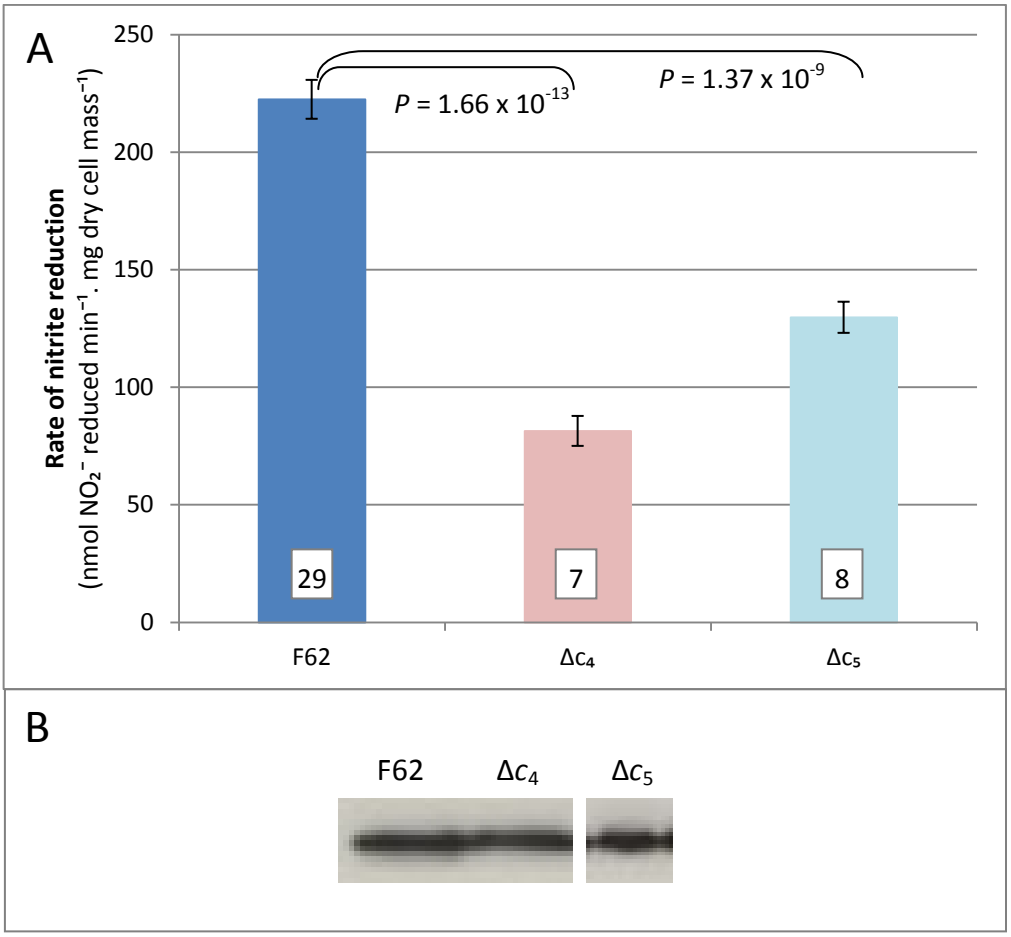


Figure 5.1 – Rates of nitrite reduction by and washed bacterial suspensions of strains F62, JCGC800 (Δ*c*$_4$) and JCGC850 (Δ*c*$_5$)

A) Rates of nitrite reduction by whole, washed cells using lactate as an electron donor. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell mass^{-1} ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein detected by Western blotting during oxygen-limited growth. Samples were taken 5 hours after the commencement of growth as described above.

```
Bjapo  262  GVMPAWEGRLDPSTIKAMAVYVHSLGGGK-----------------------------
Avine  275  GNMPAHKDFLGNDKAHLVAAYVYSLSNKATEK---------------------------
Nmeni  280  SQMPAWGHFLDKDKLHIMTAYVWGLSDKDGKAPVKKAEPAPTPAPAAEPAASAPAEAAQA
Ncine  280  SQMPAWGHFLDKDKLHIMTAYVWGLSNKDGKAPVKKAEPAPTPAPAAEPAASAPAEAAQA
Nlact  280  SQMPAWGHFLDKDKLHIMTAYVWGLSNKDGKAPVKKAEPAPTPAPAAEPAASAPAEAAQA
Ngono  280  SQMPAWGHFLDKDKLHIMTAYVWGLSNKDGKAPVKKAEPAP----AAEPAPSAPAEAAQA
             . ***    *. .. : ::.** .*..

Bjapo        ------------------------------------------------------------
Avine        ------------------------------------------------------------
Nmeni        VSEAKPAAAEPKAEEKAAPAAKADGK-----------------------------------
Ncine        ASEAKPAAAEPKAEEKAAPAAKVDGKQVYETVCAACHGNAIPGIPHVGTKADWSDRIKKG
Nlact        ASEAKPAAAEPKAEEKAAPAAKVDGKQVYETVCAACHGNAIPGIPHVGTKADWADRIKKG
Ngono        ASEAKPAAAEPKAEEKAAPAAKADGKQVYETVCAACHGNAIPGIPHVGTKADWADRIKKG


Bjapo        -------------------------------------------
Avine        -------------------------------------------
Nmeni        -------------------------------------------
Ncine        KDTLHKHAIEGFNTMPAKGGRGDLSDDEVKAAVDYMVNQSGGKF
Nlact        KDTLHKHAIEGFNTMPAKGGRGDLSDDEVKAAVDYMVNQSGGKF
Ngono        KDTLHKHAIEGFNTMPAKGGRGDLSDDEVKAAVDYMVNQSGGKF
```

Figure 5.2 – Multiple alignment of CcoP/ FixP amino acid sequences from *Bradyrhizobium japonicum*, *Azotobacter vinelandii* DJ, *Neisseria meningitidis* MC58, *Neisseria cinerea* ATCC 14685, *Neisseria lactamica* ATCC 23970 and *Neisseria gonorrhoeae* FA1090

The additional heme-binding motif is highlighted. The * indicates that the amino acid residues are identical in all sequences shown; the symbol : indicates conservative substitutions; the symbol . indicates semi-conserved substitutions.

Cys-X-X-Cys-His motif that is predicted to be the site of covalent attachment of a heme group. The function of this third heme group was unknown. However, a comparison of the third heme-binding domain of CcoP with other proteins in the Neisserial database revealed that residues 269-439 of CcoP are 63.1% similar to residues 114-279 of cytochrome c_5 (Figure 5.3). As cytochrome c_5 was previously shown to be a potential electron donor to AniA in the gonococcus, it was speculated that the third heme group of the gonococcal CcoP protein might also transfer electrons to outer membrane redox proteins such as AniA.

5.4 Site-directed mutagenesis of the third heme-binding domain of cytochrome CcoP

Multiple attempts by co-workers to delete the entire gene encoding cytochrome CcoP failed, suggesting that the *ccoP* gene is essential for gonococcal survival (Tovell, 2007). As it was impossible to delete the *ccoP* gene, site-directed mutagenesis was used to mutagenise the third potential heme-binding site of CcoP (see Chapter 2 for method). Strains AHGC100 (*kan*^{R} control), AHGC101 (CcoP C368A), AHGC102 (CcoP C371A), AHGC103 (CcoP C371A H372A) and AHGC104 (CcoP C368A C371A H372A) were constructed in order to determine whether this heme-binding domain is required for cytochrome oxidase activity, or whether it provides a pathway for electron transfer to AniA. Four different combinations of mutations were constructed in order to ensure that at least one combination would prevent the binding of heme at the third CXXCH amino acid motif of CcoP. When multiple mutations in the CcoP coding sequence were required, the frequency of isolating transformants containing the correct combination of mutations was lower. For example, the proportion of transformants containing the correct combination of mutations was low for strain AHGC104 (C368A C371A

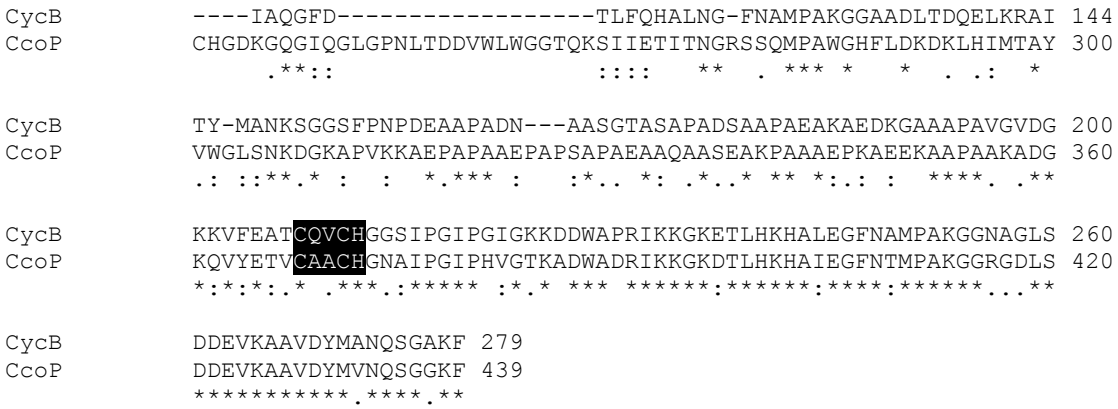


Figure 5.3 – Pairwise alignment of the gonococcal CcoP/CycB amino acid sequences

Sequence similarity between the third heme-binding domain of CcoP and the second heme-binding domain of cytochrome c_5 is shown. The second and third heme-binding motifs of CycB and CcoP, respectively, are highlighted. The * indicates that the amino acid residues are identical in all sequences shown; the symbol : indicates conserved substitutions; the symbol . indicates semi-conserved substitutions.

H372A), which required the highest number of mutations, but much higher for strain AHGC101 (C368A), which required fewer mutations. The *c*-type cytochrome complement of the resulting CcoP third heme group mutants was checked to confirm that heme could no longer bind to the third heme group of CcoP. Cultures of strains AHGC100, AHGC101, AHGC102, AHGC103 and AHGC104 were grown under oxygen-limited conditions supplemented with nitrite and samples of these cultures were taken after 7 hours. In strains AHGC101, AHGC102, AHGC103 and AHGC104, the band corresponding to cytochrome CcoP was lighter than in strain AHGC100, and breakdown products were present (Figure 5.4). This confirmed that in all four mutants the third heme group of CcoP had been altered and was unable to bind heme.

5.5 Effects of substitutions in the *C*-terminal heme-binding domain of CcoP on aerobic growth and respiration

As CcoP is required for gonococcal respiration, it was possible that mutagenesis of the third heme group of this protein would affect aerobic growth and oxygen reduction rates of the gonococcus. To test this, strains AHGC100, AHGC101, AHGC102, AHGC103 and AHGC104 were grown under oxygen-sufficient growth conditions. The growth of all strains with mutagenised heme groups was identical to the AHGC100 control strain, suggesting that the third heme group of CcoP is not essential for oxygen-sufficient growth (Figure 5.5A). Indeed, the rates of oxygen reduction by all strains were almost identical to the AHGC100 control strain, confirming that the third heme group of CcoP is not required for oxygen respiration (Figure 5.5B).

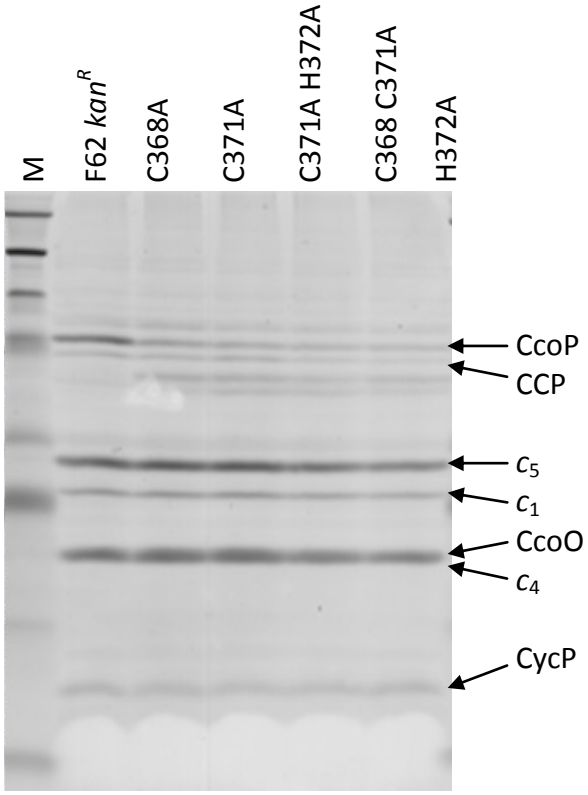


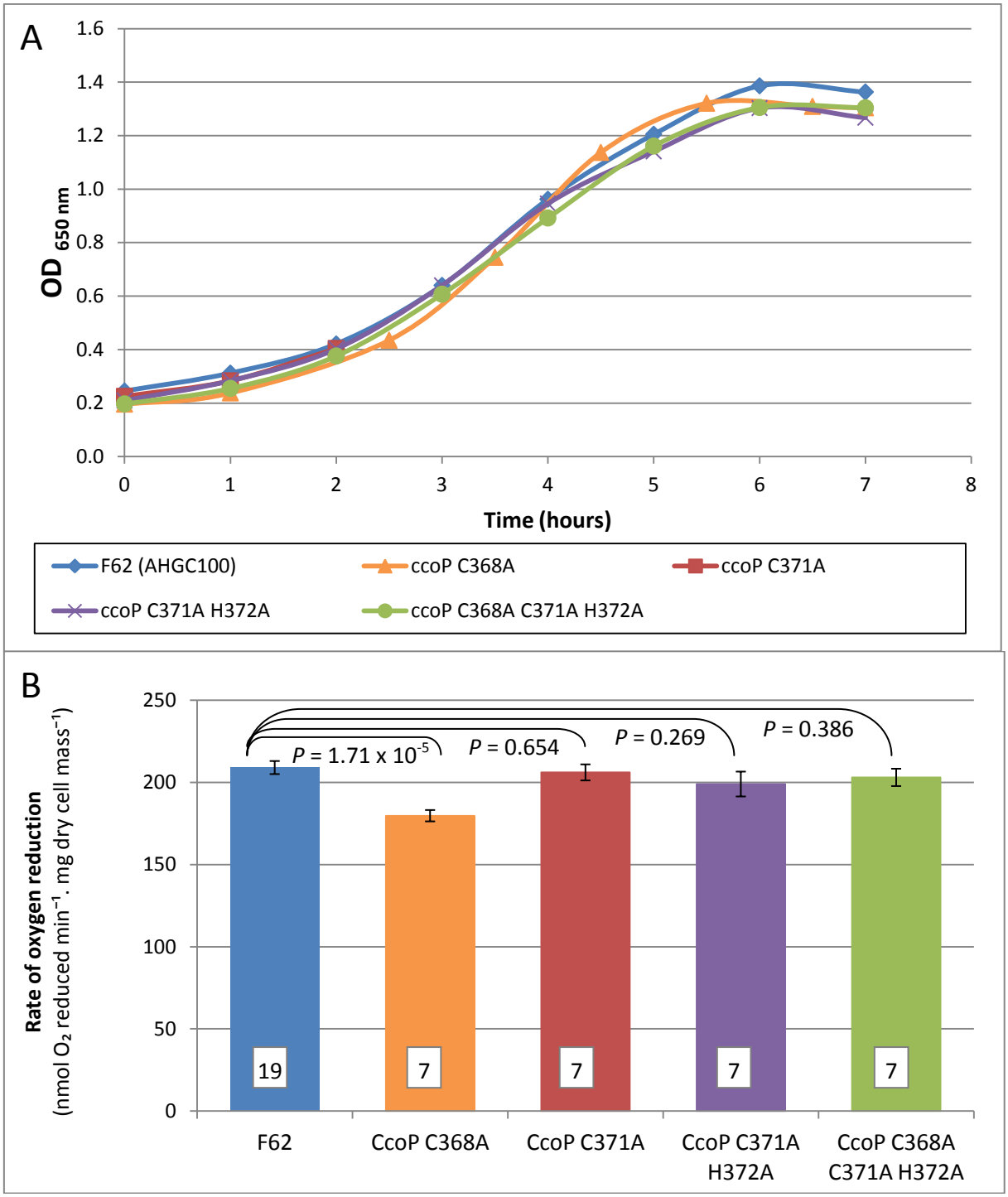
Figure 5.4 – SDS-PAGE gel stained for covalently bound heme in CcoP third heme group mutants

Lane 1 shows protein marker; lane 2 shows strain AHGC100 (labelled F62); lane 3 shows strain AHGC101 (labelled C368A); lane 4 shows strain AHGC102 (labelled C371A); lane 5 shows strain AHGC103 (labelled C371A H372A); and lane 6 shows strain AHGC104 (labelled C368A C371A C372A). Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 5 mM sodium nitrite after 1 hour and 2 hours of growth, respectively. After 7 hours of growth, samples of bacteria were harvested, lysed and the proteins separated by SDS-PAGE and stained for covalently bound heme. Note that the CcoO band is a doublet with cytochrome c_4.

Figure 5.5 – Aerobic growth and rates of oxygen reduction by CcoP third heme group mutants

A) Aerobic growth of strains AHGC100 (F62 *kan*^{R}), AHGC101 (CcoP C368A), AHGC102 (CcoP C371A), AHGC103 (CcoP C371A H372A) and AHGC104 (CcoP C368A C371A C372A). Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Rates of oxygen reduction by washed bacterial suspensions of the above strains. Cultures were grown under oxygen-sufficient conditions until the optical density at 650 nm reached a value between 0.8-1. Bacteria were then harvested, washed and their ability to reduce oxygen was measured using a Clark-type oxygen electrode. Rates are nmol of oxygen reduced min^{-1}. (mg of bacterial biomass) $^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.


A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 650 nm
0
1
2
3
4
5
6
7
8
Time (hours)
F62 (AHGC100)
ccoP C368A
ccoP C371A
ccoP C371A H372A
ccoP C368A C371A H372A
B
Rate of oxygen reduction
(nmol O2 reduced min−1. mg dry cell mass−1)
250
200
150
100
50
0
P = 1.71 x 10-5
P = 0.654
P = 0.269
P = 0.386
19
7
7
7
7
F62
CcoP C368A
CcoP C371A
CcoP C371A H372A
CcoP C368A C371A H372A

5.6 Effects of substitutions in the *C*-terminal heme-binding domain of CcoP on oxygen-limited growth and nitrite reduction

To determine whether the third heme group of CcoP is involved in electron transfer to the nitrite reductase, AniA, strains AHGC100, AHGC101, AHGC102, AHGC103 and AHGC104 were grown under oxygen-limited cultures in the presence of nitrite. The mutated strains grew slightly less rapidly and reduced nitrite more slowly than the AHGC100 control strain during the later stages of growth (Figure 5.6A and 5.6B). Rates of nitrite reduction by all four mutants were between 30% and 50% slower than that of the AHGC100 control strain, suggesting that the third heme group of CcoP is an electron donor to AniA (Figure 5.7A). Similar quantities of AniA had accumulated in all five strains, confirming that the slower rates of nitrite reduction by the mutants were due to decreased rates of electron transfer to AniA rather than to the synthesis of lower, rate-limiting amounts of the terminal nitrite reductase, AniA (Figure 5.7B).

5.7 Attempted transfer of the CcoP C368A mutation into a single mutant defective in cytochrome c_5

To determine whether CcoP and cytochrome c_5 were perhaps the only two electron donors to AniA, attempts were made to transfer the c_5 deletion mutation into the CcoP third heme group mutant. Strain AHGC101 was piliated and transformed with linear DNA created by PCR using primers YLC5AUF and YLC5CDR and pYL7 as a template. The AHGC101 strain was also transformed with linear DNA encoding an *fnr*::*ermC* deletion mutation as a positive control. Although transformants were recovered, complete recombination at the cytochrome c_5

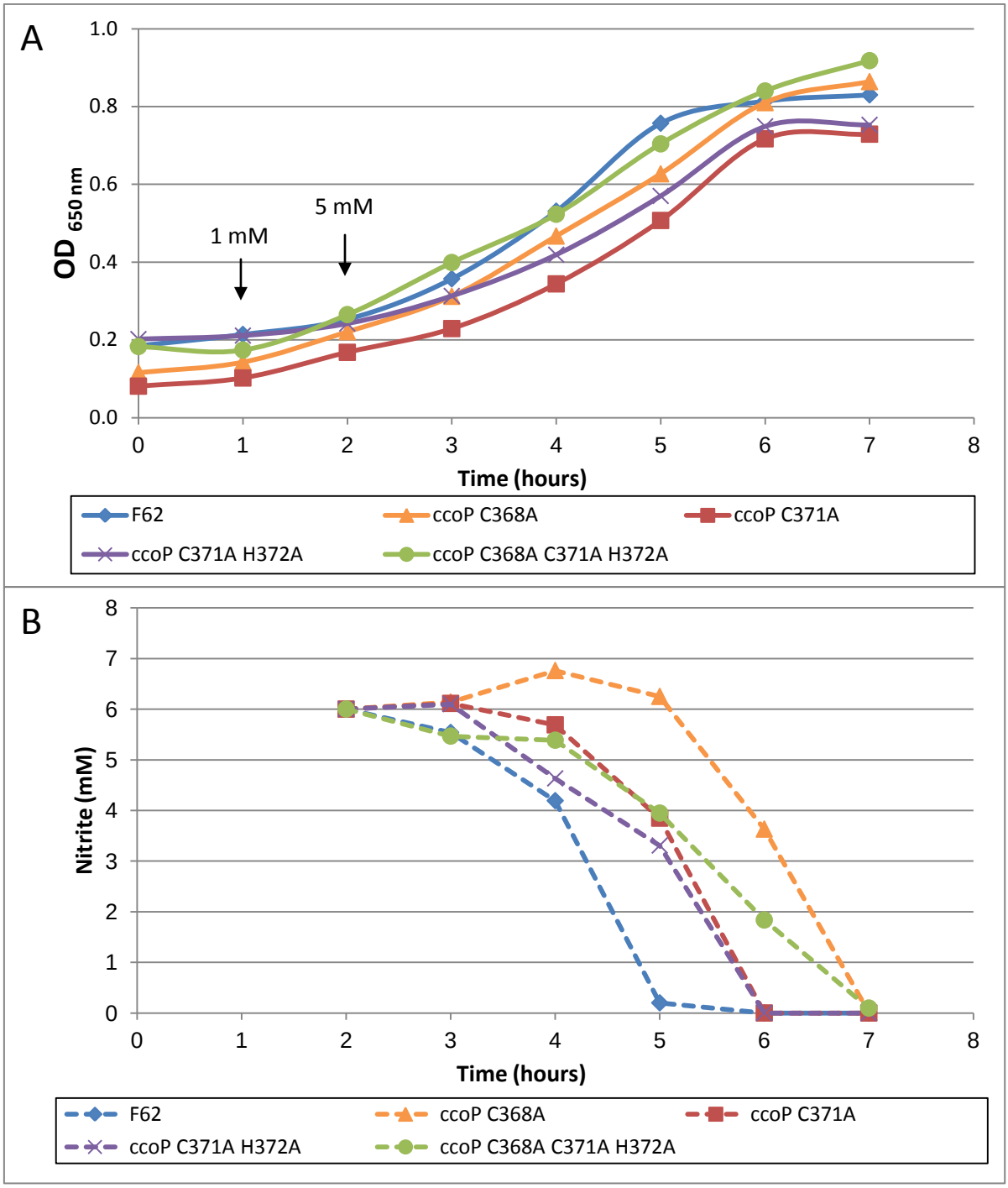


Figure 5.6 – Oxygen-limited, nitrite-supplemented growth of CcoP third heme group mutants

A) Oxygen-limited growth of strains AHGC100 (F62 *kan*^{R}), AHGC101 (CcoP C368A), AHGC102 (CcoP C371A), AHGC103 (CcoP C371A H372A) and AHGC104 (CcoP C368A C371A C372A). Cultures were grown under oxygen-limited conditions supplemented with sodium nitrite (indicated by arrows). Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Concentration of nitrite remaining in growth medium for cultures shown in Figure 5.6A.

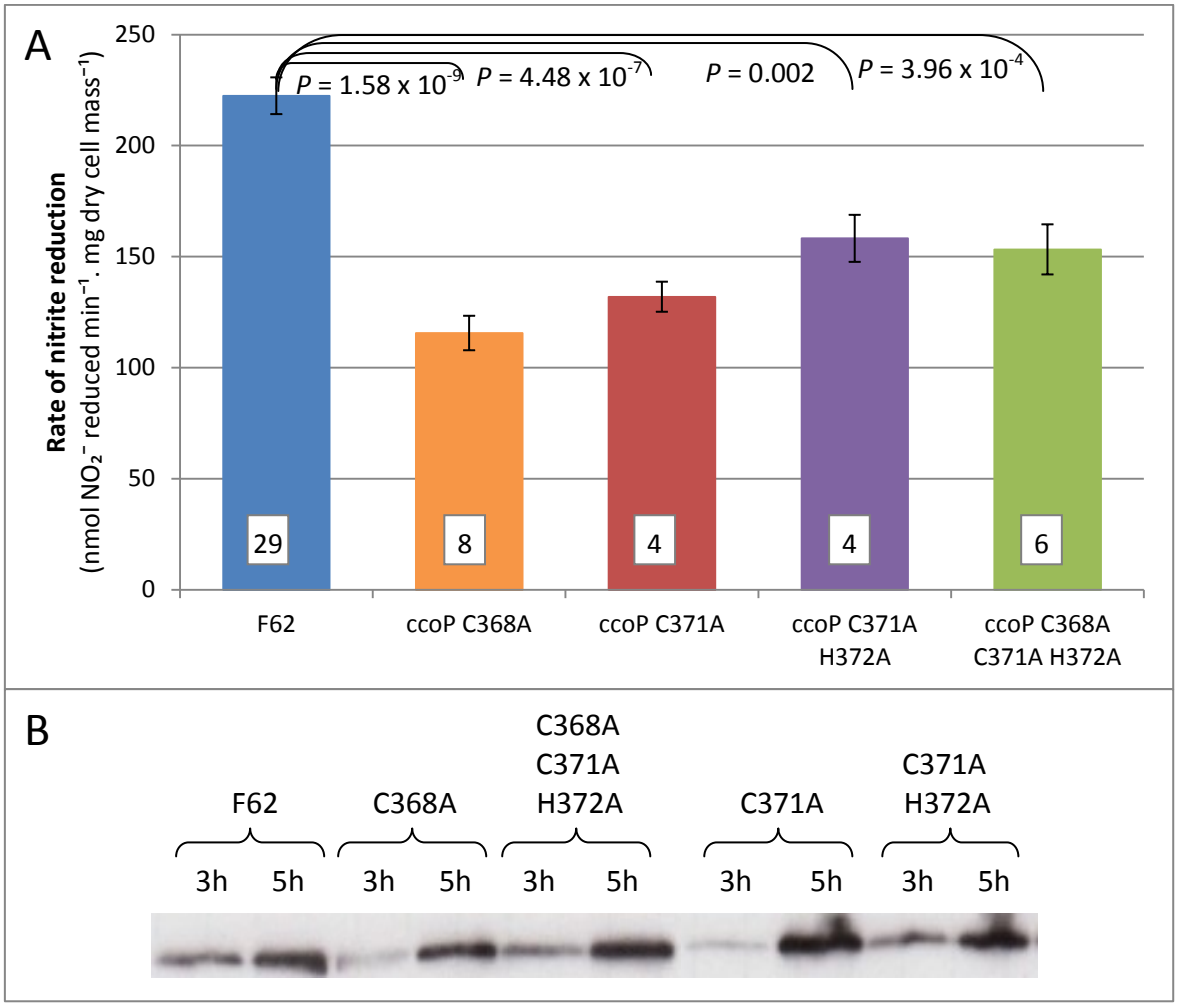


Figure 5.7 – Rates of nitrite reduction and accumulation of AniA protein in CcoP third heme group mutants

A) Rates of nitrite reduction by washed bacterial suspensions of strains F62, AHGC101 (*ccoP* C368A), AHGC102 (*ccoP* C371A), AHGC103 (*ccoP* C371A H372A) and AHGC104 (*ccoP* C368A C371A C372A) using lactate as an electron donor. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein during oxygen-limited growth detected by Western blotting. Samples from strains F62 (labelled F62), AHGC101 (labelled C368A), AHGC104 (labelled C368A C371A C372A), AHGC102 (labelled C371A) and AHGC103 (labelled C371A H372A) were taken at 3 hours and 5 hours after the commencement of growth experiments.

locus was unsuccessful as candidates for the double mutant had both the wild-type cytochrome c_5 gene and the deleted copy replaced with the erythromycin cassette present on the chromosome. It is believed that these copies were most likely located adjacent to one another on the chromosome. Multiple attempts to complete this construction were unsuccessful, implying that this is a lethal combination of mutations. Cytochrome c_5 has two heme groups, the second of which is homologous to the third heme group of CcoP. One could speculate that the incompatibility of the CcoP third heme group and c_5 mutations could be due to a build up of reactive oxygen species generated by the lower flux of electrons to the terminal oxidase as a result of the loss of cytochrome c_5. To overcome this problem, a truncated form of the cytochrome c_5 gene that included only the first heme-binding domain was constructed. This construct was transformed into the parent strain and strain AHGC101 to produce strains JCGC310 and JCGC320, respectively. Strains F62, JCGC310 (c_5Δ200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c_5Δ200-279) were grown under oxygen-limited conditions supplemented with sodium nitrite. The truncated cytochrome c_5 mutant and the CcoP mutant lacking its third heme group both grew at a slower rate compared to the parent strain, but at similar rates to each other (Figure 5.8A). These two mutants also reduced nitrite at a similar rate to each other (Figure 5.8B). However, the combination of these two mutations resulted in a decreased growth rate and nitrite reduction rate compared to its single mutant constituents. The compounding effect of the removal of the second domain of cytochrome c_5 and the third heme group of CcoP suggests that these two domains provide independent routes of electron transfer to AniA. The *c*-type cytochrome complements of strains JCGC310 (c_5Δ200-279) and JCGC320 (CcoP C368A c_5Δ200-279) were checked to confirm that they were able to synthesise only the

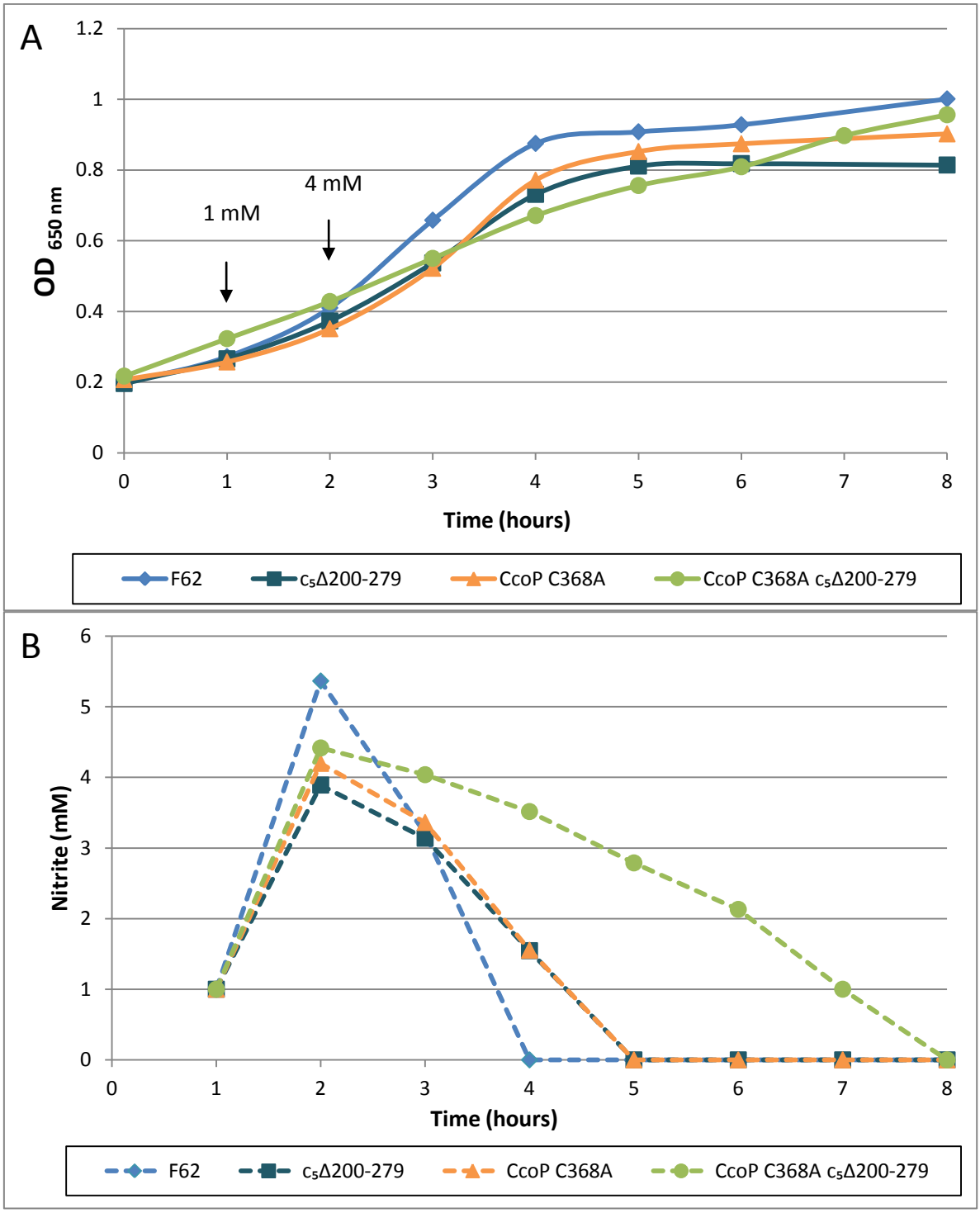


Figure 5.8 – Oxygen-limited, nitrite-supplemented growth of CcoP third heme group mutants

A) Growth of strains F62, JCGC310 (c_5Δ200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c_5Δ200-279). Arrows indicate the addition of sodium nitrite to cultures. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Concentration of nitrite remaining in growth medium for cultures shown in Figure 5.8A.

truncated version of cytochrome c_5. Proteins from samples of these cultures were separated by SDS-PAGE and stained for the presence of covalently bound heme. For strains JCGC310 (c_5Δ200-279) and JCGC320 (CcoP C368A c_5Δ200-279), the band corresponding to cytochrome c_5 was absent, and a band of approximately 20 kDa (corresponding to the predicted size of the truncated form of cytochrome c_5) was present (Figure 5.9A and 5.9B). In strains AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c_5Δ200-279), the band corresponding to CcoP was stained less heavily than in the F62 strain, confirming that the third heme binding group of CcoP was disrupted in these strains (Figure 5.9A and 5.9B). The rates of nitrite reduction of the parent strain, the single truncated c_5 mutant (JCGC310), the CcoP-c_5 double mutant (JCGC320) and the *aniA* deletion mutant (JCGC200) were measured using the whole cell nitrite reduction assay. Unlike other nitrite reduction assays described previously, cultures were supplemented with a lower concentration of nitrite during growth. This was because of the higher sensitivity of strains JCGC320 and JCGC200 to 5 mM sodium nitrite, the addition of which resulted in decreased growth rates and in some cases the cessation of growth. For this set of nitrite reduction assays, cultures were supplemented during growth with 0.1 mM and 1 mM sodium nitrite 1 h and 2 h post-inoculation. The rate of nitrite reduction by strain JCGC310 was lower than that of strain JCGC850, which lacks the entire cytochrome c_5 gene (Figures 5.1 and 5.10A). This could suggest that the first domain of cytochrome c_5 might somehow "steal" electrons from an alternative electron transfer pathway to AniA, and by deleting the whole cytochrome c_5 gene this is prevented. However, there is not sufficient information to fully interpret this phenomenon. Interestingly, the rate of nitrite reduction by a double mutant lacking the third heme group of CcoP and the second domain of cytochrome c_5 was 88% lower than the parent strain (Figure 5.10A). This rate of

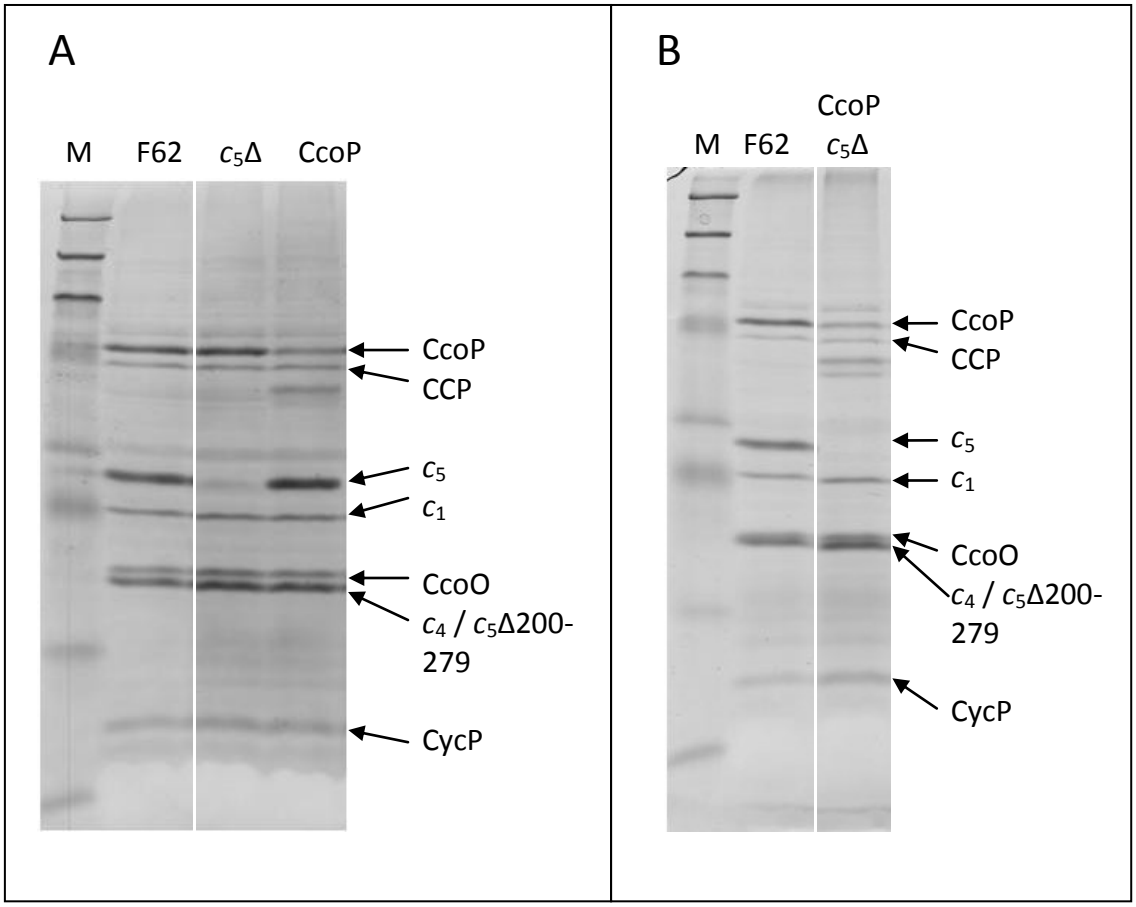


Figure 5.9 – SDS-PAGE gels stained for the presence of covalently bound heme

A) The presence of covalently bound heme in strains F62, JCGC310 (labelled c_5Δ) and AHGC101 (labelled CcoP). Cultures were grown under oxygen-limited conditions as described in Figure 5.8. Samples were taken 5 hours after the commencement of growth, then lysed and the proteins separated by SDS-PAGE and stained for covalently bound heme.

B) The presence of covalently bound heme in strains F62 and JCGC320 (labelled CcoP c_5Δ). Samples were prepared as above. Note that although in Figure 5.4 bands representing cytochromes CcoO and c_4 overlapped, here they are separated. Note also that bands representing cytochromes c_4 and the truncated cytochrome c_5 (c_5Δ200-279) overlap in these figures.

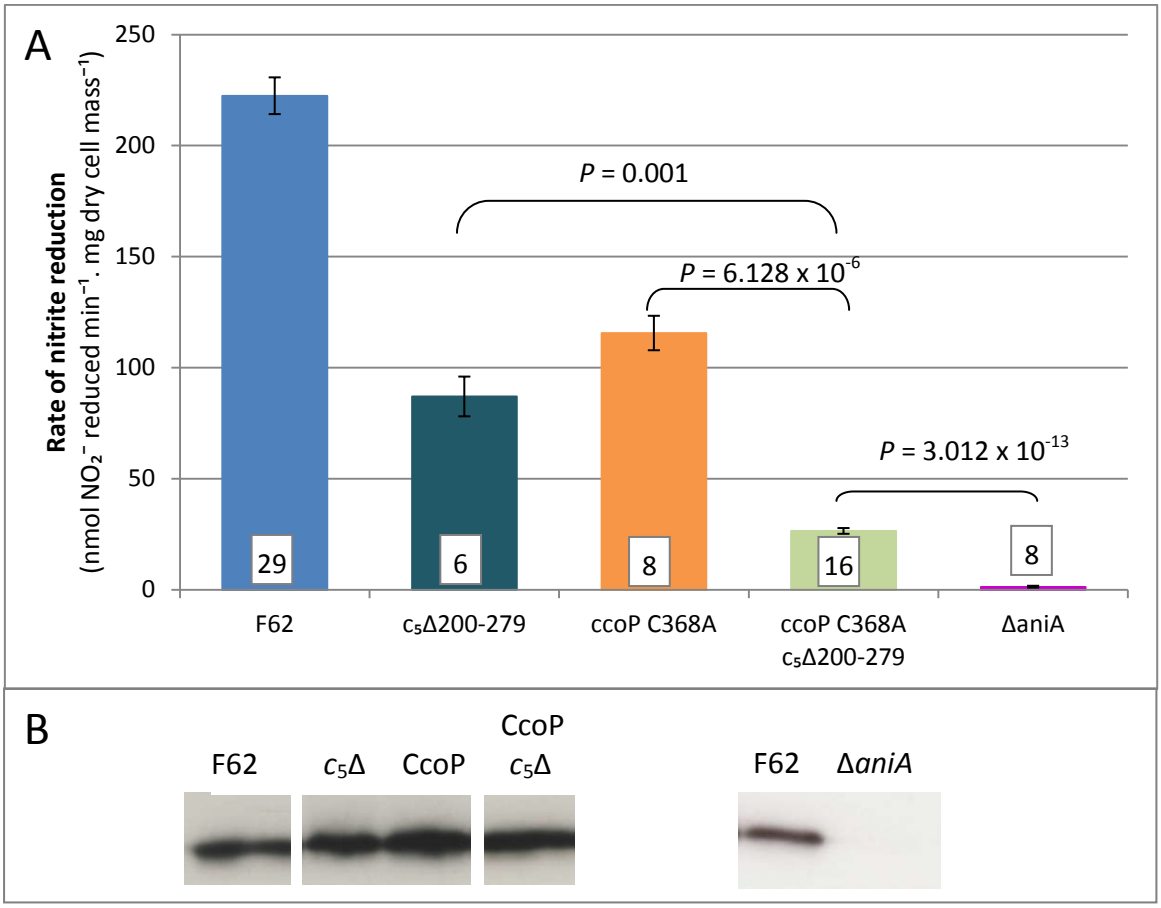


Figure 5.10 – Rates of nitrite reduction and accumulation of AniA protein in strains F62, JCGC310 (c_5Δ200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c_5Δ200-279)

A) Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC310, AHGC101 and JCGC320. Cultures were grown under oxygen-limited conditions supplemented with 0.1 mM and 1 mM sodium nitrite 1 h and 2 h post-inoculation. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein in the above strains. Cultures were grown under oxygen-limited conditions as described above, and samples were taken 5 hours after the commencement of growth experiments. Note that no AniA accumulates in an *aniA* deletion mutant.

nitrite reduction is significantly lower than the rate of nitrite reduction of either single mutant on its own. The same amount of AniA protein had accumulated in the CcoP-c_5 double mutant compared to the F62 parent strain, as shown by a Western blot (Figure 5.10B). This confirmed that the decrease in nitrite reduction rate was not due to a lower accumulation of AniA in the CcoP-c_5 double mutant. The results of these assays suggest that the third heme group of CcoP and the second heme group of cytochrome c_5 form parallel pathways of electron transfer to AniA, and that these pathways provide the majority of electrons for the reduction of nitrite.

To determine whether the second heme group of cytochrome c_5 was involved in electron transfer to the terminal oxidase, the truncated cytochrome c_5 mutant (strain JCGC310) was grown under aerobic conditions and assayed for its ability to reduce oxygen. The rate of oxygen reduction by the truncated cytochrome c_5 mutant was the same as that of the parent strain (Figure 5.11), implying that the two heme-binding domains of cytochrome c_5 might function independently.

5.8 Transfer of the cytochrome c_4 deletion mutation into the CcoP C368A mutant

In an attempt to decrease the nitrite reduction capacity of the gonococcus, a deletion mutation of the cytochrome c_4 gene was introduced into strain AHGC101 to create strain JCGC340. Strains F62, AHGC101 (CcoP C368A), JCGC800 (Δc_4), and JCGC340 (CcoP C368A Δc_4) were grown under oxygen-limited, nitrite-supplemented conditions. The growth rate of the CcoP C368A Δc_4 double mutant was slower than both of the single mutants, and nitrite was

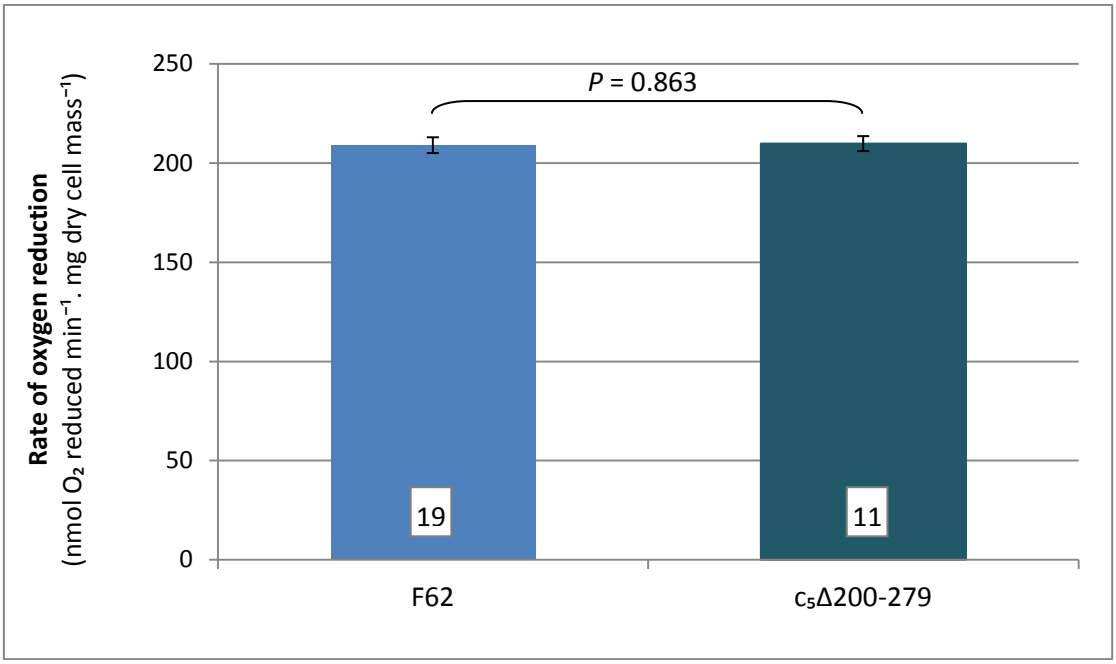


Figure 5.11 – Rates of oxygen reduction by washed bacterial suspensions of strains F62 and JCGC310 (c_5Δ200-279)

Rates of oxygen reduction by washed bacterial suspensions of the above strains. Cultures were grown under oxygen-sufficient conditions until the optical density at 650 nm reached a value between 0.8-1. Bacteria were then harvested, washed and their ability to reduce oxygen was measured using a Clark-type oxygen electrode. Rates are nmol of oxygen reduced min^{-1}. (mg of bacterial biomass) $^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

lost from the culture at a slower rate (Figure 5.12A and B). The rate of nitrite reduction by strain JCGC340 (CcoP C368A Δc_4) was 27% that of the parent strain, which is lower than the rate of nitrite reduction by the cytochrome c_4 single mutant and the CcoP single mutant lacking the third heme group (Figure 5.13). This suggests that the third heme group of CcoP and cytochrome c_4 might lie on different electron transfer pathways to AniA. Western analysis revealed that the same abundance of AniA was present in all of the aforementioned cultures, verifying that the differences in nitrite reduction rates were not due to a lower accumulation of the AniA protein (Figure 5.12C).

5.9 Transfer of the cytochrome c_2 deletion mutation into a CcoP C368A Δc_4 double mutant

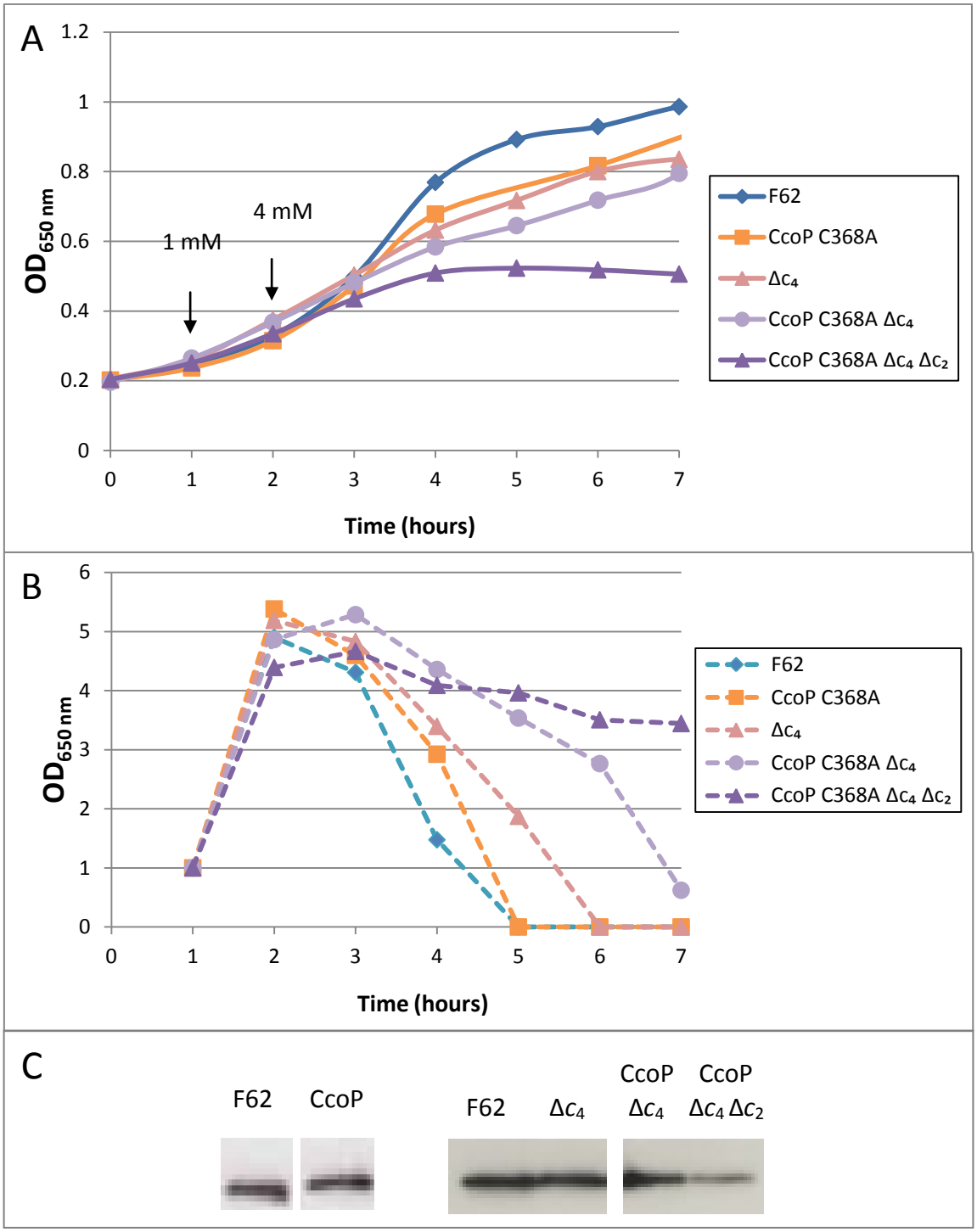
It has been shown in previous chapters that cytochrome c_2 is required for nitrite reduction to take place at its maximum rate. To determine whether cytochrome c_2 forms part of the same pathway of electron transfer to AniA as the CcoP or cytochrome c_4 pathways, the cytochrome c_2 deletion mutation was transferred into strain JCGC340 (CcoP C368A Δc_4) to produce strain JCGC351 (CcoP C368A Δc_4 Δc_2). Oxygen-limited, nitrite-supplemented growth of this strain was compared to that of strains F62, AHGC101 (CcoP C368A), JCGC800 (Δc_4) and JCGC340. The deletion of cytochrome c_2 from strain JCGC340 (CcoP C368A Δc_4) severely decreased the growth rate of this strain, and after 5 hours the cell density began to decline (Figure 5.12A). The poor growth of this culture prevented the rate of nitrite reduction of strain JCGC351 from being measured quantitatively, even when cultures were supplemented with lower concentrations of sodium nitrite. However, it can be noted from the loss of nitrite

Figure 5.12 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc_2), JCGC800 (Δc_4), JCGC340 (CcoP C368A Δc_4) and JCGC351 (CcoP C368A Δc_4 Δc_2)

A) Growth of the above strains under oxygen-limited, nitrite-supplemented conditions. Arrows indicate the addition of sodium nitrite to cultures. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Concentration of nitrite remaining in growth medium for cultures shown in Figure 5.12A.

C) Accumulation of AniA protein in the above strains. Samples were taken 5 hours after the commencement of growth experiments.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Time (hours)
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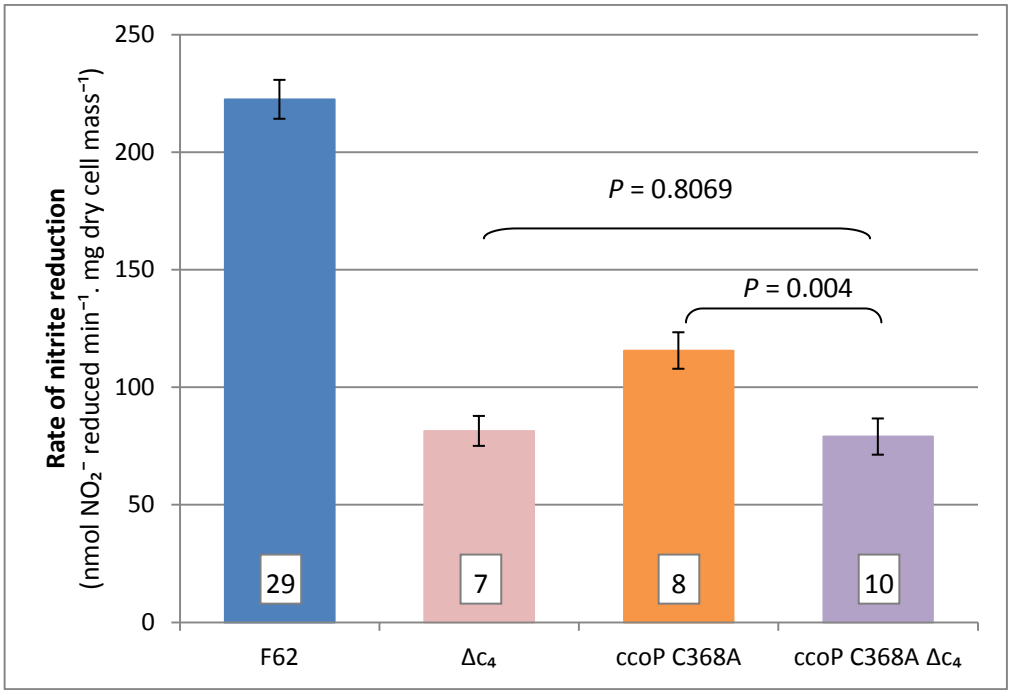


Figure 5.13 – Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC800 (Δ*c*$_4$), AHGC101 (CcoP C368A) and JCGC340 (CcoP C368A Δ*c*$_4$)

Rates of nitrite reduction by whole, washed cells using lactate as an electron donor. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell mass^{-1} ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

from the media that the rate of nitrite reduction of strain JCGC351 during growth was much slower than strain JCGC340 (Figure 5.12B). This indicates that the deletion of cytochrome c_2 from a strain lacking the third heme group of CcoP and the cytochrome c_4 is extremely detrimental, and suggests that cytochrome c_2 might form a separate pathway from the CcoP and cytochrome c_4 pathways. Unexpectedly, Western analysis revealed that AniA did not accumulate in the CcoP C368A Δc_4 Δc_2 triple mutant to the same extent as the parent strain (Figure 5.12C). As the expression of AniA is activated by FNR, which is only active under low oxygen tensions, this suggests that oxygen is not being efficiently removed from the media by the triple mutant. This would mean that the bacteria would be unable to reduce any nitrite in the media, the toxicity of which could be resulting in the termination of growth of the triple mutant (Figure 5.12A).

5.10 Addition of mutations to the CcoP C368A c_5 Δ200-279 double mutant

The double mutant defective in the third heme group of CcoP (CcoP C368) and the second domain of cytochrome c_5 (c_5 Δ200-279) retained a nitrite reduction rate of 12%. To determine whether any remaining pathways of electron transfer originated from the branch point at the cytochrome bc_1 complex, the myxothiazol sensitivity of strain JCGC320 (CcoP C368A c_5 Δ200-279) was tested. Myxothiazol inhibits electron transfer through the cytochrome bc_1 complex, so if any remaining pathways of electron transfer to AniA receive electrons from cytochrome c_1, the rate of nitrite reduction will be decreased on the addition of this inhibitor. In the presence of myxothiazol, the parental rate of nitrite reduction was decreased by 75%, confirming that the routes of electron transfer to AniA pass through the cytochrome bc_1 complex (Figure 5.14A). The rate of nitrite reduction by strain JCGC320 was

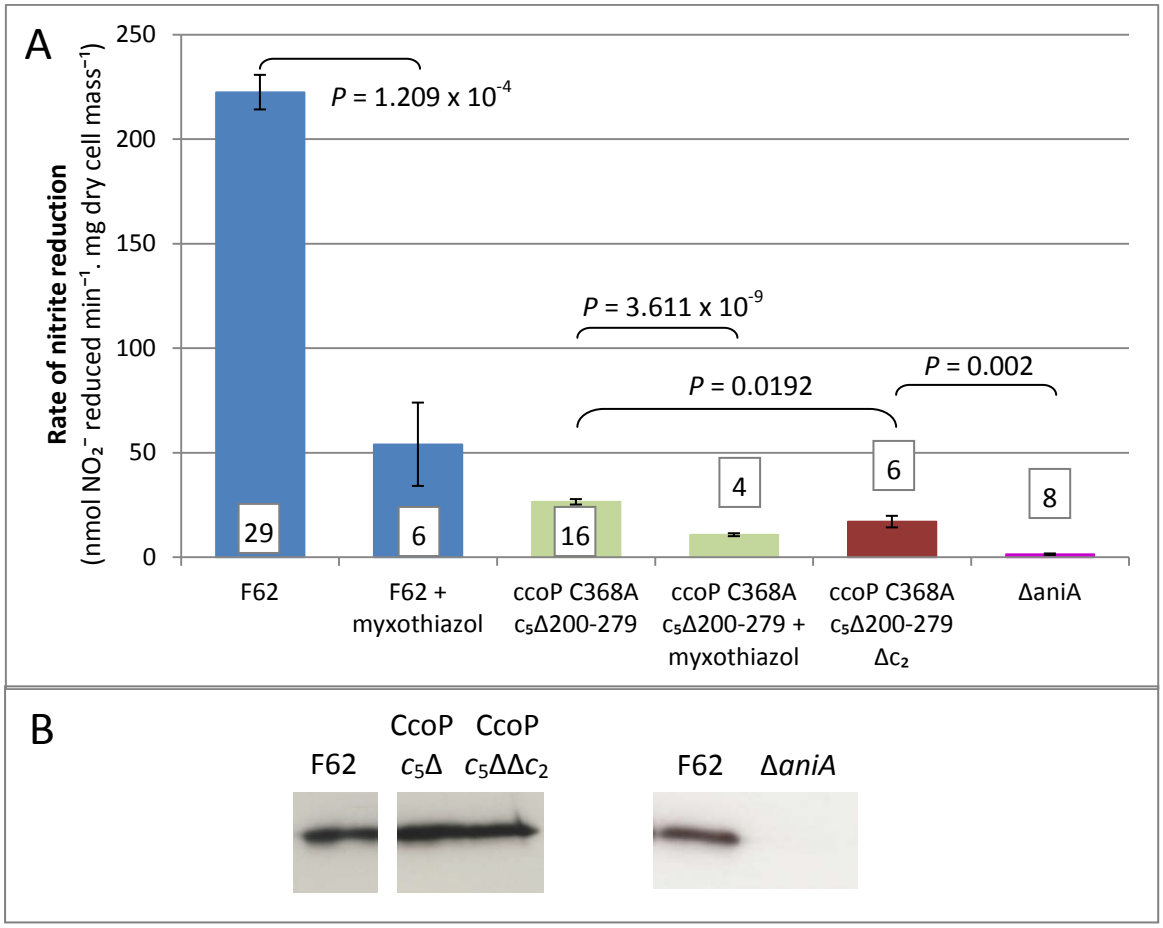


Figure 5.14 – Rates of nitrite reduction and accumulation of AniA protein in strains F62, JCGC320 (CcoP C368A c_5Δ200-279), JCGC350 (CcoP C368A c_5Δ200-279 Δc_2) and JCGC200 (Δ*aniA*) in the presence and absence of 1 µM myxothiazol

A) Rates of nitrite reduction by washed bacterial suspensions of the strains listed above. The rates of nitrite reduction of strains F62 and JCGC320 were measured in the presence and absence of 1 µM myxothiazol. Cultures were grown under oxygen-limited conditions supplemented with 0.1 mM and 1 mM sodium nitrite 1 h and 2 h post-inoculation. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

B) Accumulation of AniA protein in the strains listed above. Cultures were grown under oxygen-limited conditions as described above, and samples were taken 5 hours after the commencement of growth experiments.

decreased by 59% in the presence of myxothiazol, suggesting that at least one of any remaining electron donors to AniA receives electrons from the cytochrome bc_1 complex (Figure 5.14A).

It was reported in Chapter 4 that cytochrome c_2 might be an electron donor to AniA. It could therefore be speculated that the residual nitrite reduction activity of the double mutant defective in the third heme group of CcoP and the second domain of cytochrome c_5 could involve cytochrome c_2. A triple mutant, strain JCGC350, defective in the third heme group of CcoP, the second domain of cytochrome c_5 and the entire cytochrome c_2 gene was therefore constructed. The rate of nitrite reduction by the triple mutant was 40% lower than the CcoP-c_5 double mutant rate (Figure 5.14A), and the level of accumulation of AniA was identical in all strains tested (Figure 5.14B). The difference between the nitrite reduction rates of strains JCGC320 and JCGC350 was statistically significant (*P*-value = 0.0192). This implies that cytochrome c_2 could provide a pathway of electron transfer to AniA that is independent from the second heme group of cytochrome c_5 and the third heme group of CcoP.

5.11 Cytochrome *c*′ as a potential electron donor to AniA

As well as cytochromes CcoP, c_5, c_4 and c_2, additional redox proteins are encoded in the gonococcus. Cytochrome *c*′ (encoded by the *cycP* gene) is a 14 kDa lipoprotein attached to the outer membrane. It binds nitric oxide, which can be produced internally from gonococcal respiration or externally from host defence mechanisms or from other commensal organisms. As cytochrome *c*′ is constitutively expressed, it is believed to protect the g200gonococcus from nitric oxide while the nitric oxide reductase, NorB, is being

synthesized (Turner *et al.*, 2005). Although the gonococcal cytochrome *c*′ has been characterised, it is possible that it could also function as an electron donor to AniA.

To determine whether cytochrome *c*′ is required for oxygen-limited growth, strains F62 and JCGC214 (Δ*cycP*) were grown under oxygen-limited conditions supplemented with sodium nitrite. The growth rate of the strain lacking cytochrome *c*′ was lower than the parent strain, and nitrite was used at a slower rate by cultures of this mutant (Figure 5.15A). The rate of nitrite reduction by bacteria lacking cytochrome *c*′ was 46% lower than that of the parent strain (Figure 5.15B), and the level of accumulation of AniA was identical in both strains (Figure 5.15C). This suggested that cytochrome *c*′ could be an electron donor to AniA, and that CycP might be responsible for the residual nitrite reduction activity of strain JCGC320 (CcoP C368A c_5Δ200-279). The rate of nitrite reduction of strain JCGC355 (CcoP C368A c_5Δ200-279 Δ*cyc*P) was 9% that of the parent strain (Figure 5.15B), and the same level of AniA protein accumulated in the triple mutant (Figure 5.15C). Although the rate of nitrite reduction by strain JCGC355 was lower than strain JCGC320, this difference was not statistically significant (*P*-value = 0.078). This suggests that cytochrome *c*′ does not provide electrons for the residual nitrite reduction activity observed in strain JCGC320.

5.12 The lipid-associated azurin, Laz, as a potential electron donor to AniA

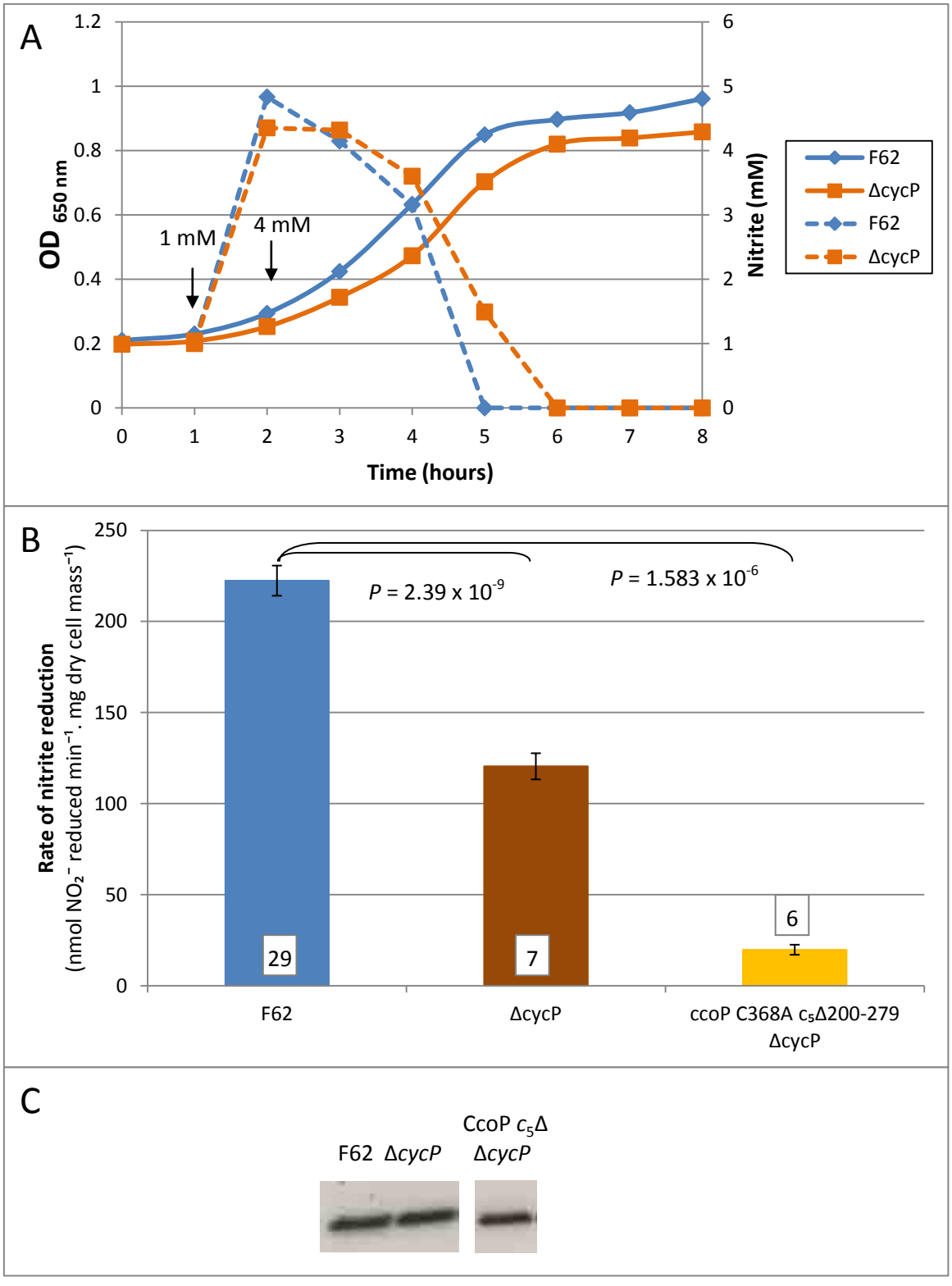
Another redox protein that could reduce AniA is the lipid-associated azurin, Laz. Azurins are blue-copper proteins that contain a type I copper site, and in other organisms are often electron donors to nitrite reductases. For example, *Paracoccus denitrificans* encodes a cytochrome *c* nitrite reductase to which the azurin donates electrons

Figure 5.15 – Oxygen-limited growth and nitrite reduction during growth of strains F62, JCGC214 (Δ*cycP*) and JCGC355 (CcoP C368A c_5Δ200-279 Δ*cycP*)

A) Growth of strains F62 and JCGC214. Solid lines indicate the $OD_{650\ nm}$ of cultures, and dotted lines indicate the concentration of nitrite present in the media. Arrows indicate the addition of sodium nitrite to cultures. Growth curves shown are representative of duplicate cultures from two independent growth experiments.

B) Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC214 and JCGC355. Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean (indicated by error bars). The numbers in boxes indicate the number of replicates, which were measured from at least two biologically replicated cultures.

C) Accumulation of AniA protein in strains F62, JCGC214 and JCGC355. Samples were taken after 5 hours of growth.


A
1.2
1
0.8
0.6
0.4
0.2
0
OD 650 nm
6
5
4
3
2
1
0
Nitrite (mM)
1 mM
4 mM
0
1
2
3
4
5
6
7
8
Time (hours)
F62
ΔcycP
F62
ΔcycP
B
Rate of nitrite reduction
(nmol NO2- reduced min-1. mg dry cell mass-1)
250
200
150
100
50
0
P = 2.39 x 10-9
P = 1.583 x 10-6
29
7
6
F62
ΔcycP
ccoP C368A c5Δ200-279
ΔcycP
C
F62 ΔcycP
CcoP c5Δ
ΔcycP

(Moir & Ferguson, 1994; Koutny *et al.*, 1999; Pearson *et al.*, 2003). Azurins can also reduce copper-containing nitrite reductases such as the NirK homologue found in *Pseudomonas aureofaciens* (Zumft *et al.*, 1987). Unlike azurins found in other bacteria, the gonococcal azurin is lipid-associated and targeted to the outer membrane (Gotschlich & Seiff, 1987; Kawula *et al.*, 1987). Although it has been shown to be important in protecting the gonococcus against hydrogen peroxide (Wu *et al.*, 2005), there has been no evidence so far to suggest that it is involved in the electron transfer chain to nitrite.

Strains F62 and JCGC890 (Δ*laz*) were grown under oxygen-limited conditions supplemented with sodium nitrite. Strain JCGC890 reduced nitrite at a rate of 84% that of the parent strain (Table 5.1), and the level of accumulation of AniA in both strains was identical, showing that the decreased rate of nitrite reduction was not due to a lower accumulation of AniA (Figure 5.16). Although the difference in nitrite reduction rate between the parent strain and JCGC890 is significant, one would expect a more pronounced decrease in nitrite reduction rate if Laz were an important electron donor to AniA. To determine whether Laz was a major pathway of electron transfer to AniA, the Laz gene was deleted from other deletion mutant strains. The rates of nitrite reduction by cultures of strains JCGC892 (Δc_2 Δ*laz*), JCGC335 (*ccoP*C368A Δ*laz*), JCGC317 (c_5Δ200-279 Δ*laz*) and JCGC352 (*ccoP*C368A c_5Δ200-279 Δ*laz*) were measured quantitatively. In all cases, the deletion of Laz from single and double mutant strains did not decrease their rate of nitrite reduction (Table 5.1). This suggests that Laz is not an electron donor to AniA, or at least plays only a minor role in the electron transfer chain to nitrite.

Table 5.1 – Rates of nitrite reduction by various mutants in combination with a *laz* deletion mutation

Strain	Rate of nitrite reduction	No. replicates	*P*-value
Parent (F62)	222 ± 8	29	
Δ*laz*	188 ± 7	7	0.004 (F62)
Δc_2	115 ± 7	19	
Δc_2 Δ*laz*	121 ± 8	6	0.557 (Δc_2)
***ccoP* C368A**	115 ± 8	8	
ccoP* C368A Δ*laz	114 ± 8	6	0.9351 (*ccoP* C368A)
c_5 Δ200-279	87 ± 9	6	
c_5 Δ200-279 Δ*laz*	64 ± 10	6	0.1214 (c_5 Δ200-279)
***ccoP* C368A c_5 Δ200-279**	26 ± 1.3	16	
ccoP* C368A c_5 Δ200-279 Δ*laz	27 ± 1	6	0.6744 (*ccoP* C368A c_5 Δ200-279)

Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC890 (Δ*laz*), JCGC851 (Δc_2), JCGC892 (Δc_2 Δ*laz*), AHGC101 (*ccoP* C368A), JCGC335 (*ccoP* C368A Δ*laz*), JCGC310 (c_5Δ200-279), JCGC317 (c_5Δ200-279 Δ*laz*), JCGC320 (*ccoP* C368A c_5Δ200-279) and JCGC352 (*ccoP* C368A c_5Δ200-279 Δ*laz*). Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean. The number of replicates is indicated, and were measured from at least two biologically replicated cultures. *P*-values are relative to the strain shown in brackets.

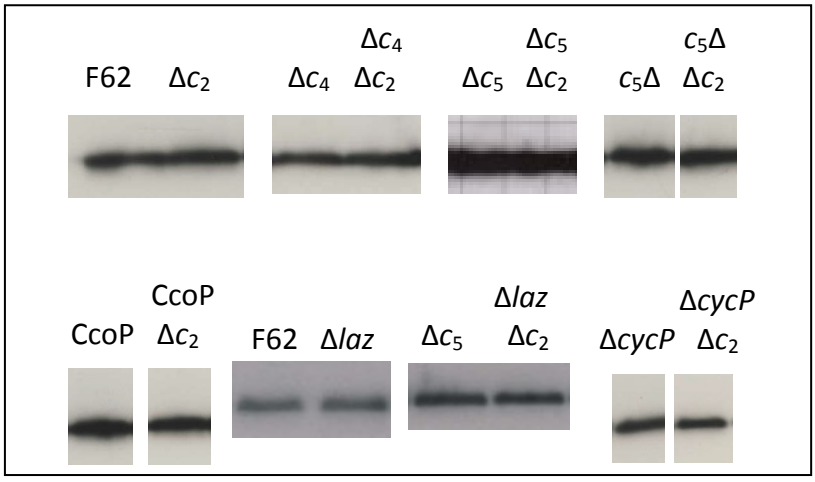


Figure 5.16 – Accumulation of AniA protein in various mutants in combination with a cytochrome c_2 mutation

Accumulation of AniA protein in strains F62 (labelled F62), JCGC851 (labelled Δc_2), JCGC800 (labelled Δc_4), JCGC853 (labelled Δc_4 Δc_2), JCGC850 (labelled Δc_5), JCGC852 (labelled Δc_5 Δc_2), JCGC310 (labelled c_5Δ), JCGC315 (labelled c_5Δ Δc_2), AHGC101 (CcoP), JCGC330 (CcoP Δc_2), JCGC890 (Δ*laz*), JCGC892 (Δ*laz* Δc_2), JCGC214 (Δ*cycP*) and JCGC216 (Δ*cycP* Δc_2) during oxygen-limited growth detected by Western blotting. Samples were taken after 5 hours of growth.

5.13 Role of cytochrome c_2 in electron transfer to AniA

When the cytochrome c_2 coding region was deleted from strains JCGC340 (CcoP C368A Δc_4) and JCGC320 (CcoP C368A Δc_5) to produce strains JCGC351 and JCGC350, respectively, the deletion of cytochrome c_2 resulted in a decreased nitrite reduction rate in both strains. This suggests that the cytochrome c_2 electron transfer pathway to AniA is independent of the CcoP, cytochrome c_4 and cytochrome c_5 pathways. To further dissect the electron transfer pathways to AniA, cytochrome c_2 deletion mutations were added to strains JCGC800 (Δc_4), JCGC850 (Δc_5), JCGC310 (c_5 Δ200-279) and AHGC101 (CcoP C368A) to produce strains JCGC853 (Δc_4 Δc_2), JCGC852 (Δc_5 Δc_2), JCGC315 (c_5 Δ200-279 Δc_2) and JCGC330 (CcoP C368A Δc_2), respectively. The deletion of cytochrome c_2 from strains JCGC850 (Δc_5) and JCGC310 (c_5Δ200-279) resulted in a 66% and 60% decrease in nitrite reduction rate, respectively (Table 5.2). This suggests that the cytochrome c_2 pathway of electron transfer to AniA is independent of the cytochrome c_5 pathway. The deletion of cytochrome c_2 from strain JCGC800 (lacking cytochrome c_4) resulted in a slightly increased rate of nitrite reduction, but the difference was not statistically significant (Table 5.2). This suggests that cytochromes c_2 and cytochrome c_4 form part of the same electron transfer pathway. A similar phenomenon was seen in the mutant lacking the third heme-binding motif of CcoP (strain AHGC101). Deleting the cytochrome c_2 gene from this strain has no effect on the rate of nitrite reduction (Table 5.2), supporting the conclusion that CcoP is involved in the same electron transfer pathway as cytochrome c_2. The same abundance of AniA was present all in cultures tested, verifying that the differences in nitrite reduction rates were not due to a lower accumulation of the AniA protein in cultures (Figure 5.16).

Table 5.2 – Rates of nitrite reduction by various mutants in combination with a cytochrome c_2 mutation

Strain	Rate of nitrite reduction	No. replicates	*P*-value
Parent (F62)	222 ± 8	29	
Δc_2	115 ± 7	19	1.37 x 10^{-12} (F62)
Δc_4	81 ± 6	7	1.66 x 10^{-13} (F62)
Δc_4 Δc_2	93 ± 5	8	0.17 (Δc_4)
Δc_5	130 ± 7	8	1.37 x 10^{-9} (F62)
Δc_5 Δc_2	86 ± 9	11	9.136 x 10^{-4} (Δc_5)
c_5Δ200-279	87 ± 9	6	1.26 x 10^{-8} (F62), 0.003 (Δc_5)
c_5Δ200-279 Δc_2	52 ± 4	6	0.01 (c_5Δ200-279)
***ccoP* C368A**	115 ± 8	8	1.58 x 10^{-9} (F62)
***ccoP* C368A Δc_2**	115 ± 15	5	0.972 (CcoP C368A)
Δ*laz*	188 ± 7	7	0.004 (F62)
Δ*laz* Δc_2	122 ± 8	6	0.012 (Δ*laz*)
Δ*cycP*	121 ± 7	7	2.39 x 10^{-9} (F62)
Δ*cycP* Δc_2	115 ± 6	6	0.52 (Δ*cycP*)

Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC851 (Δc_2), JCGC800 (Δc_4), JCGC853 (Δc_4 Δc_2), JCGC850 (Δc_5), JCGC852 (Δc_5 Δc_2), JCGC310 (c_5Δ200-279), JCGC315 (c_5Δ200-279 Δc_2), AHGC101 (CcoP C368A), JCGC330 (CcoP C368A Δc_2), JCGC890 (Δ*laz*), JCGC892 (Δ*laz* Δc_2), JCGC214 (Δ*cycP*) and JCGC216 (Δ*cycP* Δc_2). Cultures were grown under oxygen-limited conditions supplemented with 1 mM and 2 mM sodium nitrite after 1 and 2 hours of growth, respectively. Bacteria were harvested once cultures had reached an optical density at 650 nm of between 0.5 and 0.7 (after about 5 hours of growth). Bacteria were washed and assayed for their ability to reduce 1 mM sodium nitrite using the quantitative nitrite reduction assay. Rates are nmol of nitrite reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean. The number of replicates is indicated, and were measured from at least two biologically replicated cultures. *P*-values are relative to the strain shown in brackets.

5.14 Discussion

5.14.1 The third heme group of CcoP as an electron donor to AniA

The presence of a *C*-terminal extension on the CcoP subunit of gonococcal cytochrome cbb_3 oxidase made it a probable candidate to replace the small, soluble *c*-type cytochromes or blue copper proteins of the azurin family that transfer electrons to NirK in other bacteria. It has previously been reported that a CcoO–CcoN complex is catalytically active in oxygen reduction without the involvement of CcoP (de Gier *et al.*, 1996; Zufferey *et al.*, 1996; Arslan *et al.*, 2000). It is therefore not surprising that the third heme group of CcoP is not required for the oxygen reduction function of cytochrome cbb_3 oxidase. It is, however, required for maximum rates of nitrite reduction by the gonococcus, and this is not due to secondary effects of defects in oxygen reduction or in the synthesis of the terminal nitrite reductase, AniA. There is precedence for the dual function of a subunit of a terminal reductase. The nitrate reductase complex of *Thermus thermophilus* includes a di-heme *c*-type cytochrome, NarC, that transfers electrons from the NADH dehydrogenase to nitrite and nitric oxide reductases, bypassing the cytochrome bc_1 complex (Cava *et al.*, 2008). Loss of the third heme group of CcoP resulted in only partial loss of ability to reduce nitrite; consequently, one or more other pathways of electron transfer to AniA must be present in the gonococcus. This study has provided the first example of a component of cytochrome oxidase that plays a physiologically significant role in nitrite reduction (Hopper *et al.*, 2009). The homology of the third heme-binding domain of CcoP to the second heme-binding domain of cytochrome c_5 suggests that the origin of the *C*-terminal extension of CcoP could be a gene duplication event. It is possible that the cytochrome c_5 gene was duplicated and recombined

downstream of the CcoP locus. The majority of members of the *Neisseria* genus contain this extended CcoP protein, with the exception of *Neisseria meningitidis*. It seems that this species has lost the *C*-terminal extension to CcoP, due to a mutation resulting in a premature stop codon and therefore truncation of CcoP to a di-heme protein (Aspholm *et al.*, 2010). In all gonococcal isolates found to date, the nitrite reductase AniA is always present in the genome. This is in contrast to meningococcal isolates, many of which seem to lack the AniA protein and retain their ability to be successful pathogens (Stefanelli *et al.*, 2008). If many isolates do not have a functional copy of AniA, they are unlikely to require many routes of electron transfer to AniA. It is therefore not surprising that the meningococcus has lost the genetic determinant to construct a trihemic CcoP protein.

5.14.2 The bifunctionality of cytochrome c_5

Cytochrome c_5 is made up of two domains, each containing a heme-binding site. It has been demonstrated in this chapter that both of its heme groups seem to function independently in different roles. Deletion of the second domain of cytochrome c_5 had no effect on the ability of the bacterium to reduce oxygen, implicating the first heme group in electron transfer to the terminal oxidase. The rate of nitrite reduction by a mutant lacking the second domain of cytochrome c_5 was similar to that of the cytochrome c_5 deletion mutant, indicating that the role of the second heme group of cytochrome c_5 is to transfer electrons to the nitrite reductase, AniA. The cytochrome bc_1 complex can be considered to be a branch point of electron transfer to either the terminal oxidase or to AniA: however, it seems that cytochrome c_5 also functions as a junction point in the electron transfer chain of the gonococcus. A model for the major routes of electron transfer to AniA is presented in Figure

5.17. According to this model, the second domain of cytochrome c_5 receives electrons from the first domain of cytochrome c_5, and transfers these directly to AniA across the periplasm. The first domain of cytochrome c_5 also transfers electrons in parallel with cytochrome c_4 to the second heme-binding domain of cytochrome CcoP. The second heme group of CcoP then reduces the third domain of CcoP, which might be able to span the periplasm and donate electrons directly to AniA. In another possible model (shown in Figure 5.18), the second heme group of the CcoP protein could transfer electrons to both the third heme group of CcoP and the second heme group of cytochrome c_5. The second heme group of CcoP would be supplied with electrons from the first heme group of cytochrome c_5 and the cytochrome c_4 protein. Although it seems unlikely that the two domains of cytochrome c_5 would function independently to such an extent, there are advantages to this arrangement. If all of the electrons to be transferred to AniA had to pass through the second heme group of CcoP, this would ensure that nitrite is not used as the terminal electron acceptor when oxygen is present. Once electrons have reached the second heme group of CcoP, the powerful oxidizing effect of oxygen would prevent any electrons being transferred to the third heme group of CcoP or the second heme group of cytochrome c_5. In this way, only the terminal electron acceptor that is most thermodynamically favourable would be used, while still allowing the gonococcus to quickly adapt to changing levels of oxygen in the environment.

5.14.3 Cytochrome c_2 as an electron donor to AniA

The third heme group of cytochrome CcoP and the second heme group of cytochrome c_5 constitute the major pathways of electron transfer to the nitrite reductase, AniA. The deletion of cytochrome c_2 from mutants defective in the full complement of *c*-type

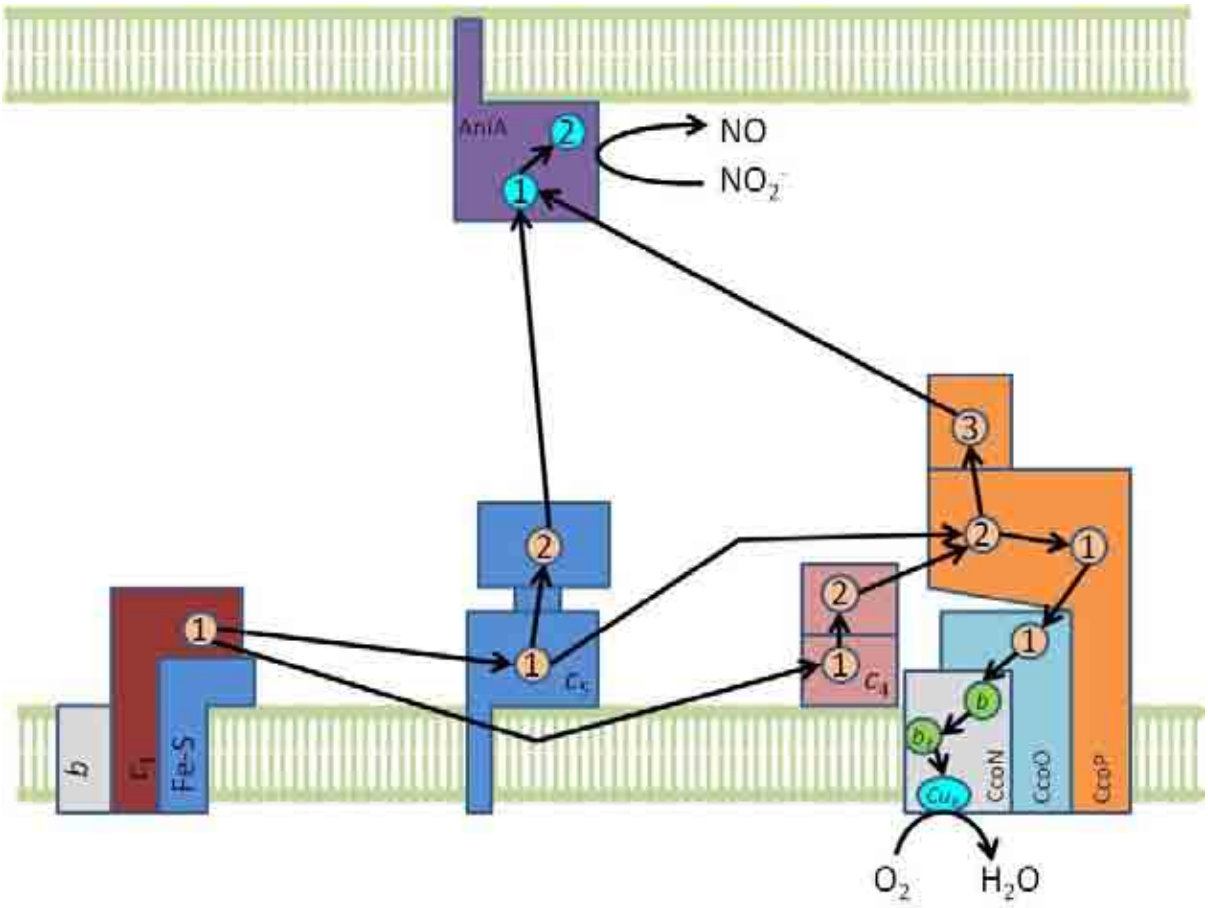


Figure 5.17 – Model of possible electron transfer pathways to the nitrite reductase, AniA

Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity.

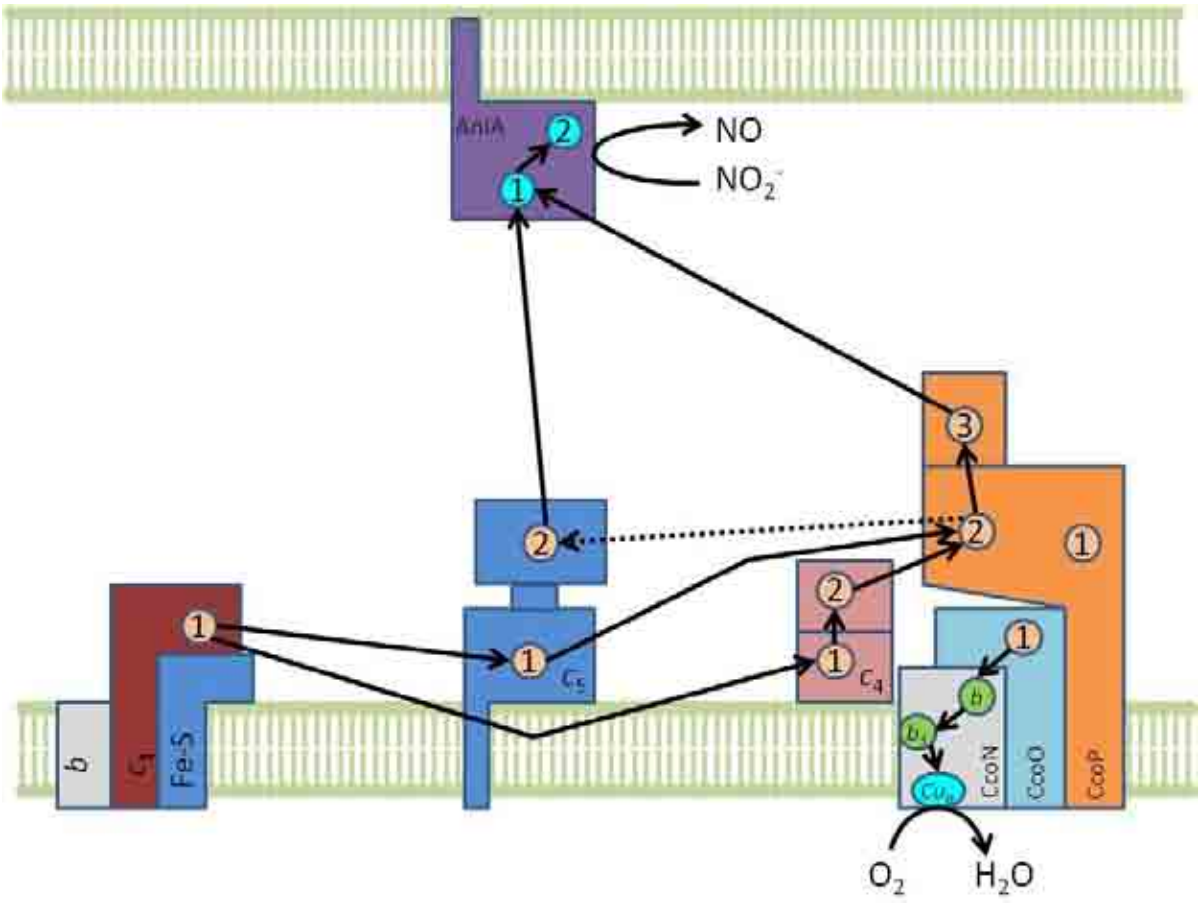


Figure 5.18 – Model of possible electron transfer pathways to the nitrite reductase, AniA

Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways, dotted lines represent potential electron transfer routes. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity.

cytochromes has either no effect or a negative effect on their ability to reduce nitrite. For example the deletion of cytochrome c_2 from strain JCGC320 (*ccoP* C368A c_5Δ200-279) decreases the nitrite reduction rate from 12% to 8% of the parental rate. It is possible that these differences in nitrite reduction rate are due to the loss of the cytochrome c_2 electron transfer pathway to AniA. This suggests that the cytochrome c_2 pathway to AniA is independent from pathways provided by cytochromes c_5, CcoP and c_4. Cytochrome c_2 might instead receive electrons directly from the cytochrome bc_1 complex (Figure 5.19).

5.14.4 Role of cytochrome c_4 in the electron transfer chain to nitrite

Although it is clear that the third heme group of CcoP and the second heme group of cytochrome c_5 account for the major pathways of electron transfer to AniA, a residual nitrite reduction activity remains. As two major components in the electron transfer chain have been removed, any remaining electron transfer to AniA could be an artefact of an imbalanced redox environment. The hypothesis that cytochrome c_4 is not a direct electron donor to AniA can be supported by the fact that cytochromes c_4 are thought to be tightly bound to membrane-integrated terminal oxidases (Abergel *et al.*, 2003; Bertini *et al.*, 2006). If this were the case in the gonococcus, electron transfer from cytochrome c_4 to AniA would be ineffective, as the distance between cytochrome c_4 and AniA would probably be too great to allow efficient electron transfer. Therefore it cannot be ruled out that cytochrome c_4 could provide a minor pathway of electron transfer to AniA under these conditions, and might account for the low residual nitrite reduction activity observed in the double mutant lacking the third heme group of CcoP and the second heme group of cytochrome c_5 (strain JCGC320).

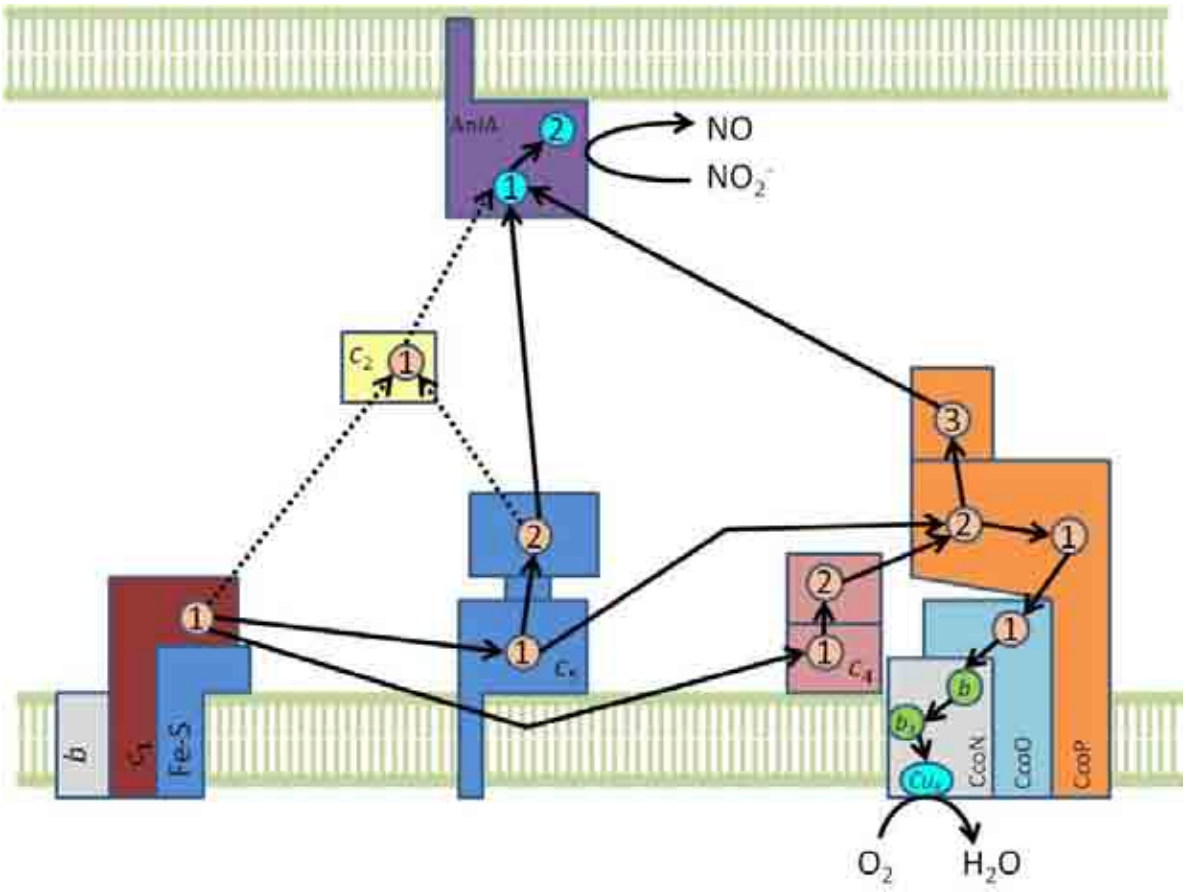


Figure 5.19 – Model of cytochrome c_2 as a possible electron donor to AniA

Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways, dotted lines represent potential electron transfer routes. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity.

Cytochrome c_4 is known to transfer electrons to the cytochrome cbb_3 complex, and, based on the solved structure of the cytochrome cbb_3-type oxidase from *Pseudomonas stutzeri*, both cytochromes c_4 and c_5 would reduce the second heme group of the CcoP subunit (Buschmann *et al.*, 2010). It is reasonable to assume that the third heme group of CcoP can be reduced by the second heme group of CcoP, as the redox centres of these domains would be in close proximity. Therefore, cytochrome c_4 and the first heme group of cytochrome c_5 contribute indirectly to electron flux to AniA via the third heme group of CcoP. It is likely that cytochrome c_4 contributes relatively more electrons to the CcoP subunit, as a proportion of the electrons that pass through cytochrome c_5 are diverted to the second heme group of cytochrome c_5, which has been shown to be an electron donor to AniA. One could therefore speculate that the decreased nitrite reduction rate in the cytochrome c_4 deletion mutant is due to the loss of a major pathway of electron transfer to the CcoP subunit, thus decreasing the electron availability to the third heme group of CcoP. The loss of cytochrome c_4 means that only the first heme group of cytochrome c_5 is available to supply electrons to the CcoP subunit. If so, fewer electrons are diverted to the third heme group of CcoP, resulting in a lower electron flux to AniA and a decreased nitrite reduction rate. It might also mean that any electrons being transferred to the second heme group of cytochrome c_5 from its first heme group are preferentially diverted to the second heme group of the CcoP subunit, and therefore fewer electrons are available to AniA via the second heme group of cytochrome c_5.

5.14.5 Poor growth of the triple mutant lacking cytochromes c_4, c_2 and the third heme group of CcoP

The accumulation of the AniA protein was the same in nearly all cultures grown under

oxygen-limited, nitrite-supplemented conditions. There were only two exceptions: the AniA deletion mutant, and a triple mutant lacking cytochromes c_4, c_2 and the third heme group of CcoP. The AniA deletion mutant lacks the *aniA* coding sequence, and therefore cannot synthesize AniA. Although the aforementioned triple mutant does synthesize AniA, the protein accumulates at a lower level compared to the parent strain. This phenomenon can be explained when we consider the properties of the deleted *c*-type cytochromes. As cytochrome c_4 is absent from this triple mutant, only cytochrome c_5 is available to provide reducing power to the terminal oxidase for oxygen reduction. As oxygen cannot be removed from media as efficiently, AniA cannot be expressed at a high level, preventing the bacterium from reducing nitrite efficiently. This means that the concentration of nitrite remains at a high level, and becomes toxic to the bacterium. The absence of the third heme group of CcoP leaves only cytochrome c_5 as the major route of electron transfer to AniA. Furthermore, the deletion of cytochrome c_2 from the double mutant lacking cytochrome c_4 and the third heme group of CcoP is incredibly detrimental to the growth of the resulting triple mutant. This suggests the role of cytochrome c_2 could be to decrease the distance between cytochrome c_5 and AniA. The absence of cytochrome c_2 would therefore reduce the efficiency of electron transfer from cytochrome c_5 to AniA. The lack of cytochromes c_4, c_2 and the third heme group of CcoP means that cytochrome c_5 alone must provide all of the electrons to both AniA and the cbb_3-type oxidase, which means that this strain struggles to reduce both oxygen and nitrite efficiently. As AniA expression is FNR-dependent, the inability to remove oxygen from the growth media results in a decreased expression of AniA, thus preventing the bacterium from reducing nitrite. The lower accumulation of AniA in the triple mutant lacking cytochromes c_4, c_2 and the third heme group of CcoP highlights the

bifunctionalism of cytochrome c_5 in the electron transfer chain of the gonococcus.

5.14.6 Cytochrome *c*′ and the lipid-associated azurin are not major electron donors to AniA

It is possible that cytochrome *c*′ could be an electron donor to AniA. Although the deletion of the *cycP* gene resulted in a decreased nitrite reduction rate, the deletion of cytochrome *c*′ from strain JCGC321 (CcoP C368A c_5Δ200-279) did not further decrease the nitrite reduction rate of this strain significantly (*P*-value = 0.078). This suggests that cytochrome *c*′ does not constitute a major pathway of electron transfer to AniA. One could speculate that the decrease in nitrite reduction rate by a *cycP* mutant could result from an increased abundance of free nitric oxide (NO) in the periplasm due to the lack of NO-binding cytochrome *c*′ (Turner *et al.*, 2005). This might decrease the rate of nitrite reduction by AniA due to product inhibition, where the high concentration of nitric oxide would prevent the enzymic reduction of nitrite by AniA. This in itself might be a nitric oxide protection system, as this would prevent the accumulation of nitric oxide to a toxic level. The localisation of cytochrome *c*′ to the outer membrane makes cytochrome *c*′ an unlikely electron donor to AniA, as it would not be large enough to span the periplasm and hence receive electrons from the inner membrane. This lends more support to the latter explanation, that the decreased nitrite reduction rate of the Δ*cycP* mutant results from product inhibition by the higher NO concentration in the periplasm.

Unlike most azurins, the gonococcal lipid-associated azurin, Laz, is localised to the outer membrane (Gotschlich & Seiff, 1987). Its small size of 18.5 kDa would be unlikely to span the

periplasm to supply AniA with electrons from the inner membrane. Therefore if Laz is an electron donor to AniA, it would probably be very inefficient and would not represent a major pathway of electron transfer to AniA. Deletion of the gene encoding Laz did decrease the nitrite reduction rate of this mutant compared to the parent strain, but had no effect on the rates of nitrite reduction of any strains lacking a full complement of *c*-type cytochromes. More importantly, the deletion of *laz* from strain JCGC321 (CcoP C368A c_5Δ200-279) did not further decrease the nitrite reduction rate of this strain significantly (*P*-value = 0.6744). This suggests that the gonococcal azurin does not form a major pathway of electron transfer to AniA, but might contribute to its reduction. This result was unexpected, as in many other bacteria azurins are major electron donors to nitrite reductases (Zumft *et al.*, 1987; Pearson *et al.*, 2003). The only published phenotype for a gonococcal Laz mutant is an increased sensitivity to hydrogen peroxide (Wu *et al.*, 2005). One could hypothesise that perhaps Laz is an electron donor to cytochrome *c* peroxidase (CCP), or is itself able to reduce hydrogen peroxide or other reactive oxygen species. It therefore seems that neither cytochrome *c*′ nor Laz can efficiently reduce the nitrite reductase, AniA.

Chapter 6

Discussion

6.1 The electron transfer chain to oxygen

Previous studies that aimed to determine the electron transfer chain to oxygen were either incorrect or incomplete. The availability of the complete genome sequence of *Neisseria gonorrhoeae* revealed that the gonococcus encodes a single terminal oxidase of cbb_3-type, a cytochrome bc_1 complex, and cytochromes c_2, c_4 and c_5, all of which were obvious candidates for electron transfer components to oxygen. It was shown in Chapter 3 that, as expected, cytochromes c_4 and c_5 form a bifurcated pathway from the cytochrome bc_1 complex to the cbb_3-type terminal oxidase (Figure 6.1). As the gonococcus has only one terminal oxidase, it was not surprising that it was impossible to delete genes for both of these cytochromes simultaneously. A c_4 c_5 double mutant could be constructed in this bacterium only when an ectopic copy of the cytochrome c_5 gene was present, the expression of which could be induced by IPTG.

The redox potentials of the gonococcal cytochromes c_4 and c_5 are unknown. However, assumptions can be made based on the redox potentials of similar *c*-type cytochromes from other organisms. Midpoint potentials can be determined experimentally using the Nernst equation:

$$E = E'_m - \frac{RT}{nF} \ln Q$$

where E = redox potential under a given set of conditions (V)
E'_m = midpoint potential at pH 7 (V)
R = gas constant (8.314 $Jmol^{-1} K^{-1}$)
T = temperature (K)
n = number of electrons
F = Faraday constant (96,485 Coulombs mol^{-1})
Q = reaction quotient

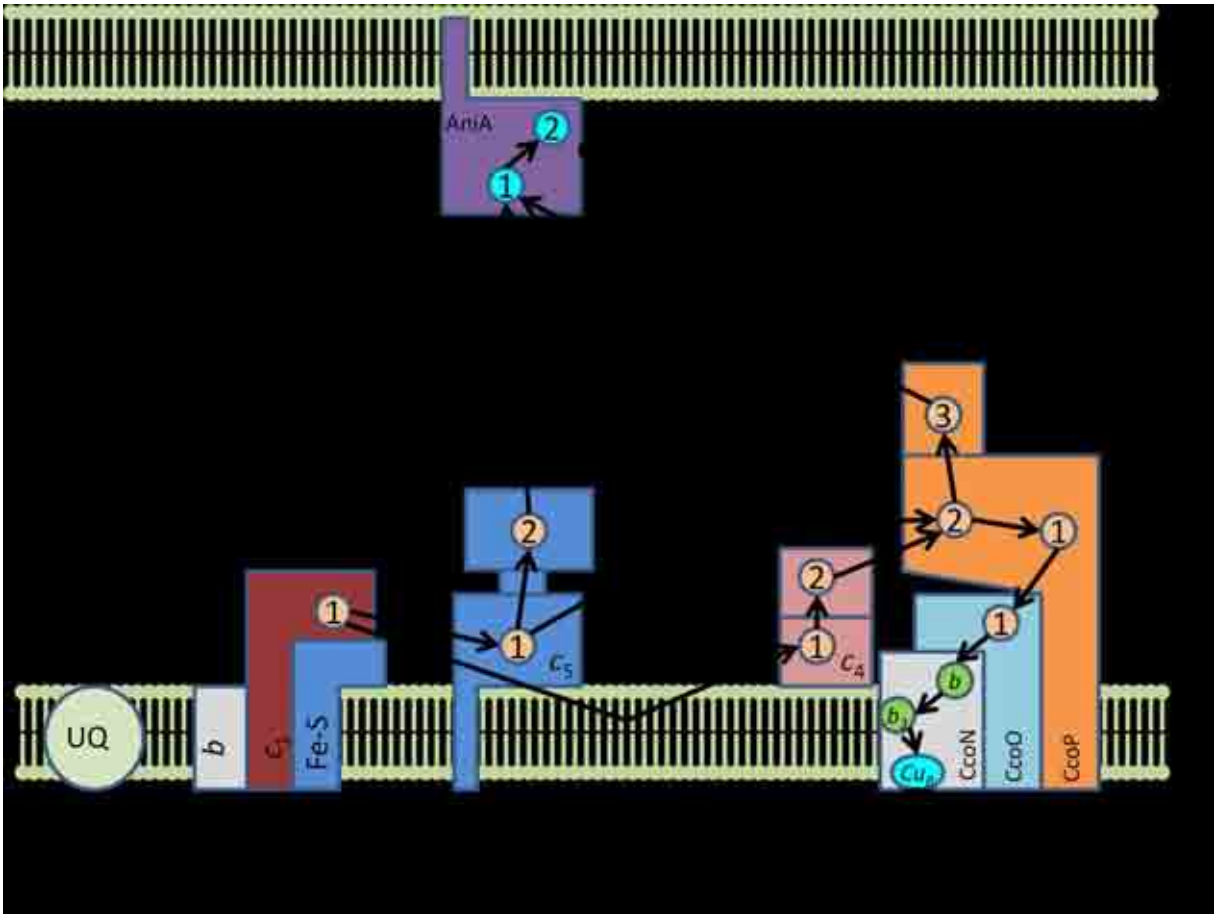


Figure 6.1 – Model of potential electron transfer pathways to the terminal reductases

Model of the electron transfer pathways to the *cbb*$_3$-type terminal oxidase and the nitrite reductase, AniA. Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways. The dimeric cytochrome *bc*$_1$ complex and trimeric AniA protein complexes are both shown as monomers for simplicity. Midpoint potentials are estimated from values in Table 6.1.

As discussed in Chapter 1, the gonococcal cytochrome c_4 is 36% and 33% identical to cytochromes c_4 from *A. vinelandii* and *V. cholerae*, respectively. The midpoint potentials of these cytochromes are + 300 mV for the whole cytochrome c_4 from *A. vinelandii*, and + 240 mV and + 340 mV for the individual heme groups of *V. cholerae* cytochrome c_4 (see Table 6.1). The first heme group of the gonococcal cytochrome c_5 is 49% identical to the whole cytochrome c_5 of *A. vinelandii*, which has a midpoint potential of + 312 mV (Table 6.1). The midpoint potential of the second heme group of the gonococcal cytochrome c_5 cannot be predicted as accurately, for although there are homologues in other bacteria, their redox potentials have not been determined. Based on the values obtained from other organisms (Table 6.1), the midpoint potentials of both cytochromes c_4 and c_5 would be of a similar magnitude to cytochrome c_1, suggesting that electron transfer might not be very efficient. One could speculate that cytochromes c_4 and c_5 might also be reduced directly by ubiquinol, as its midpoint potential is significantly lower (Table 6.1). It is possible that both ubiquinol and the cytochrome bc_1 complex are able to donate electrons to both cytochromes c_4 and c_5, which may account for the unexpectedly low level of inhibition of respiration by myxothiazol compared to other reports in the literature (N. Watmough, personal communication).

The midpoint potentials of components within the *R. sphaeroides* cytochrome cbb_3 oxidase have been determined (Table 6.1). Assuming that the redox centres of the gonococcal CcoO and CcoP subunits would have similar midpoint potentials, these values are lower than those predicted for cytochromes c_4 and c_5, suggesting that electron transfer from these cytochromes to the terminal oxidase could not occur. However, the proximity of redox centres can be sufficient driving force to allow these thermodynamically unfavourable

Table 6.1 – Midpoint potentials of redox components from various organisms

Organism	Redox protein/component	Redox couple	E'_m (mV)	Reference
Dehydrogenases				
E. coli	NADH dehydrogenase	NAD^+/NADH	- 320	(Unden & Bongaerts, 1997)
E. coli	Succinate dehydrogenase	fumarate/ succinate	+ 30	(Unden & Bongaerts, 1997)
Quinol/cytochrome bc_1 complex				
S. cerevisiae	Ubiquinol	UQ/UQH_2	+ 90	(Snyder & Trumpower, 1998; Covian *et al.*, 2004)
	b_L	b_L (+3)/b_L (+2)	- 30	
	b_H	b_H (+3)/b_H (+2)	+ 90	
	Rieske protein	$2Fe2S^+$/2Fe2S	+ 280*	
	c_1	Fe (+3)/ Fe (+2)	+ 270	
R. sphaeroides	Ubiquinol	UQ/UQH_2	+ 90	(Bowyer & Crofts, 1981; Crofts *et al.*, 2003)
	b_L	b_L (+3)/b_L (+2)	- 90	
	b_H	b_H (+3)/b_H (+2)	+ 50	
	Rieske protein	$2Fe2S^+$/2Fe2S	+ 310*	
	c_1	Fe (+3)/ Fe(+2)	+ 270	
***cbb*$_3$-type oxidase**				
R. sphaeroides	*c* (CcoO, 2xCcoP)	Fe (+3)/ Fe (+2)	+ 234 + 320 + 351	(Rauhamaki *et al.*, 2009)
	b (CcoN)	*b* (+3)/*b* (+2)	+ 418	
	b_3 (CcoN)	b_3 (+3)/b_3 (+2)	- 59	
	Cu_B (CcoN)	Cu (+2)/Cu(+1)	+ 330	
P. stutzeri	*c* (CcoP)	Fe (+3)/ Fe (+2)	+ 185,+ 105	(Pitcher & Watmough, 2004)
	c (CcoP)		+ 245, + 205	
	c (CcoO)		+ 245, + 215	
	b (CcoN)	*b* (+3)/*b* (+2)	+ 310,+ 265	
	b_3 (CcoN)	b_3 (+3)/b_3 (+2)	+ 225	
-	-	½ O_2 + $2H^+$ / H_2O	+ 820	(Pitcher & Watmough, 2004)
Copper-containing nitrite reductases (CuNiR)				
R. sphaeroides	Type 1 Cu	Cu (+2)/Cu(+1)	+ 247	(Olesen *et al.*, 1998)
	Type 2 Cu	Cu (+2)/Cu(+1)	<+ 200**	
P. chlororaphis	Type 1 Cu	Cu (+2)/Cu(+1)	+ 298	(Pinho *et al.*, 2004)
	Type 2 Cu	Cu (+2)/Cu(+1)	+ 172**	
A. xylosoxidans	Type 1 Cu	Cu (+2)/Cu(+1)	+ 255	(Leferink *et al.*, 2011)
	Type 2 Cu	Cu (+2)/Cu(+1)	+ 244 ** (+300 on NO_2^- binding)	
-	-	NO_2^-/NO	+ 375	(Berks *et al.*, 1995)
Azurins				
A. denitrificans	Type 1 Cu (Azurin)	Cu (+2)/Cu(+1)	+ 276	(Ainscough *et al.*, 1987)
P. aeruginosa	Type 1 Cu (Azurin)	Cu (+2)/Cu(+1)	+ 308	(Taniguchi *et al.*, 1980)

P. denitrificans	Type 1 Cu (Azurin)	Cu (+2)/Cu(+1)	+ 230	(Martinkus *et al.*, 1980)
***c*-type cytochromes**				
V. cholerae	cytochrome c_4	Fe (+3)/ Fe (+2)	+ 240 + 340	(Chang *et al.*, 2010)
A. vinelandii	cytochrome c_4	Fe (+3)/ Fe (+2)	+ 300	(Tissieres, 1956)
P. stutzeri	cytochrome c_4	Fe (+3)/ Fe (+2)	+ 241 + 328	(Kadziola & Larsen, 1997)
A. vinelandii	cytochrome c_5	Fe (+3)/ Fe (+2)	+ 312	(Carter *et al.*, 1985)
C. thiosulfatophilum	cytochrome c_{555}	Fe (+3)/ Fe (+2)	+ 145	(Korszun & Salemme, 1977)
S. violacea	cytochrome c_5	Fe (+3)/ Fe (+2)	+ 301	(Ogawa *et al.*, 2007)
T. thermophilus	cytochrome c_{552}	Fe (+3)/ Fe (+2)	+ 202	(Fee *et al.*, 2000)
Cytochromes *c*′				
Alcaligenes sp.	cytochrome *c*′	Fe (+3)/ Fe (+2)	+130	(Meyer *et al.*, 1986)
R. sphaeroides	cytochrome *c*′	Fe (+3)/ Fe (+2)	+30	(Meyer *et al.*, 1986)
A. tumefaciens	cytochrome *c*′	Fe (+3)/ Fe (+2)	+180	(Meyer *et al.*, 1983)
Cytochrome *c* peroxidases				
P. aeruginosa	cytochrome *c* cytochrome *c*	Fe (+3)/ Fe (+2) Fe (+3)/ Fe (+2)	+ 320 - 330	(Ellfolk *et al.*, 1983)
N. europaea	cytochrome *c* cytochrome *c*	Fe (+3)/ Fe (+2) Fe (+3)/ Fe (+2)	+ 450 - 258	(Arciero & Hooper, 1994)
-	-	H_2O_2 / $2H_2O$	+ 1770	(Bertagna *et al.*, 2001)
Other				
-	Ascorbate	dehydroascorbic acid/ascorbic acid	+ 58	(Weis, 1975)
-	TMPD	$TMPD^+$/TMPD	+ 260	(Jurtshuk *et al.*, 1975)

Midpoint potentials of various redox components from *Escherichia coli*, *Saccharomyces cerevisiae*, *Rhodobacter sphaeroides*, *Pseudomonas stutzeri*, *Pseudomonas chlororaphis*, *Alcaligenes xylosoxidans*, *Alcaligenes denitrificans*, *Pseudomonas aeruginosa*, *Paracoccus denitrificans*, *Vibrio cholerae*, *Azotobacter vinelandii*, *Chlorobium thiosulfatophilum*, *Shewanella violacea*, *Thermus thermophilus*, *Alcaligenes* sp., *Agrobacterium tumefaciens* and *Nitrosomonas europaea*.

*This value decreases once bound ubiquinol is fully oxidised (Snyder *et al.*, 1999).

**This value increases in the presence of nitrite to allow efficient electron transfer from Type 1 Cu centre to Type 2 Cu centre (Olesen *et al.*, 1998; Leferink *et al.*, 2011).

electron transfers to occur (Page *et al.*, 1999; Alric *et al.*, 2006). Such reactions have been found in many electron transfer chains studied to date (Alric *et al.*, 2006). This indicates that cytochromes c_4 and c_5 would have to be in close proximity to the cytochrome cbb_3 oxidase, perhaps forming a supercomplex, for efficient electron transfer to occur.

Although cytochrome c_2 did not appear to be involved in electron transfer to the terminal oxidase, the deletion of cytochrome c_2 resulted in an increased oxygen reduction rate compared to the parent strain (Table 3.1). This suggested that the loss of the cytochrome c_2 pathway diverted more electrons to cytochromes c_4 and c_5, thus increasing the electron flux to the terminal oxidase and increasing the rate of oxygen reduction. The increase in electron flux through the cytochrome bc_1 complex as a result of the deletion of cytochrome c_2 might have made the inhibition of the cytochrome bc_1 complex by myxothiazol more inefficient, which would explain the decreased sensitivity of the cytochrome c_2 mutant to myxothiazol (Table 3.1).

It has been reported that, in the meningococcus, cytochromes c_4 and c_x are electron donors to the cbb_3-type oxidase, and that c_5 is the only electron donor to AniA (Deeudom *et al.*, 2008). However, in the gonococcus, the deletion of both pathways to the terminal oxidase, i.e. cytochromes c_4 and c_5, was impossible as only one terminal oxidase is present in this bacterium. As the meningococcus also only encodes one terminal oxidase, the fact that both cytochromes c_4 and c_x can be simultaneously deleted while retaining the ability to reduce oxygen implies that these cytochromes do not form the only electron transfer pathways to the cytochrome cbb_3 complex. This suggests that cytochrome c_5 might also donate electrons

to the terminal oxidase in the meningococcus. Furthermore, it suggests that cytochrome c_2 of *N. gonorrhoeae* and cytochrome c_x of *N. meningitidis* could be minor electron donors to their respective cbb_3-type oxidases.

6.2 The electron transfer chain to nitrite

The four obvious candidates for electron donors to the nitrite reductase, AniA, were the lipid-associated azurin, Laz, and cytochromes c_4, c_5 and c_2. As cytochromes c_4 and c_5 were found to be required for electron transfer to the terminal oxidase, it was thought that they might not be involved in electron transfer to nitrite. However, the nitrite reduction rates of both of these single mutants was lower than that of the parent strain (Table 6.2), suggesting that these *c*-type cytochromes have multiple roles in the gonococcus. Cytochrome c_2 was also implicated as an electron donor to AniA, as deletion of the *cccA* gene resulted in a 48% decrease in nitrite reduction rate compared to the parent strain (Table 6.2). Although the deletion of the *laz* gene resulted in a decreased rate of nitrite reduction compared to the parent strain (Table 6.2), Laz did not appear to be a major electron donor to AniA. Double mutants lacking combinations of these four possible electron donors to AniA retained a nitrite reduction rate of at least 39%. This indicated that another electron transfer pathway to AniA must exist.

6.2.1 The third heme group of CcoP is a major electron donor to AniA

The discovery of the existence of a third heme-binding motif in a cytochrome CcoP was unprecedented. Furthermore, the gonococcal CcoP provided the first example of a

Table 6.2 – Summary table of nitrite reduction rates measured in Chapter 5

Strain	Rate of nitrite reduction	No. replicates	% parental rate	*P*-values
Parent (F62)	222 ± 8	29	100%	
Δ*ccoP*	115 ± 8	8	52%	1.58 x 10^{-9} (F62)
Δc_5	130 ± 7	8	59%	1.37 x 10^{-9} (F62)
c_5Δ200-279	87 ± 9	6	39%	1.26 x 10^{-8} (F62), 0.003 (Δc_5)
c_5Δ200-279 Δ*ccoP*	26 ± 1.3	16	12%	0.001 (c_5Δ200-279)
Δc_2	115 ± 7	19	52%	1.37 x 10^{-12} (F62)
Δc_2 Δc_5	86 ± 9	11	39%	9.136 x 10^{-4} (Δc_5)
Δc_2 c_5Δ200-279	52 ± 4	6	23%	0.01 (c_5Δ200-279)
Δc_2 Δ*ccoP*	115 ± 15	5	52%	0.972 (Δ*ccoP*)
Δc_2 Δ*ccoP* c_5Δ200-279	17 ± 3	6	8%	0.0192 (Δ*ccoP* c_5Δ200-279)
Δc_4	81 ± 6	7	36%	1.66 x 10^{-13} (F62)
Δc_4 Δc_2	93 ± 5	8	42%	0.17 (Δc_4)
Δc_4 Δ*ccoP*	79 ± 7.7	10	36%	0.8069 (Δc_4),0.004 (Δ*ccoP*)
Δc_4 Δ*ccoP* Δc_2	N/A	6	N/A	N/A
Δ*cycP*	121 ± 7	7	55%	2.39 x 10^{-9} (F62)
Δ*cycP* Δc_2	115 ± 6	6	52%	0.52 (Δ*cycP*)
Δ*cycP* Δ*ccoP* c_5Δ200-279	20 ± 3	6	9%	0.078 (Δ*ccoP* c_5 Δ200-279)
Δ*laz*	188 ± 7	7	84%	0.004 (F62)
Δ*laz* c_5Δ200-279	64 ± 10	6	29%	0.1214 (c_5 Δ200-279)
Δ*laz* Δc_2	122 ± 8	6	55%	0.012 (Δ*laz*)
Δ*laz* Δ*ccoP*	114 ± 8	6	51%	0.9351 (Δ*ccoP*)
Δ*laz* Δ*ccoP* c_5Δ200-279	27 ± 1	6	12%	0.6744 (Δ*ccoP* c_5 Δ200-279)
Δ*aniA*	1 ± 0.4	8	0.004%	0.002 (Δ*ccoP* c_5 Δ200-279 Δc_2)

Units for rates of nitrite reduction are nmol NO_2^- reduced min^{-1}. mg dry cell $mass^{-1}$ ± the standard error of the mean. The number of replicates is indicated, and were measured from at least two biologically replicated cultures. Δ*ccoP* = *ccoP* C368A. *P*-values are relative to the strain shown in brackets.

cytochrome oxidase component involved in electron donation to a nitrite reductase rather than to the catalytic subunit of the terminal oxidase (Hopper *et al.*, 2009). Although this work has not identified the redox proteins that supply electrons to the third heme group of cytochrome CcoP, one could speculate that the second heme group of CcoP reduces the third heme group (Figure 6.1). If the redox potential of the third heme group of CcoP (or the homologous second heme group of cytochrome c_5) were known, it may aid in the prediction of the electron donor to the third heme group of CcoP. The fact that cytochromes c_4 and c_5 are electron donors to terminal oxidase indicates that they are able to reduce the second heme group of CcoP, and therefore can be considered as indirect electron donors to AniA via the third heme group of CcoP.

6.2.2 The second heme group of cytochrome c_5 is a major electron donor to AniA

The rate of nitrite reduction by a cytochrome c_5 deletion mutant was 59% that of the parent strain (Table 6.2), implicating cytochrome c_5 as an electron donor to AniA. The third heme-binding domain of CcoP is similar to the second heme-binding domain of cytochrome c_5, which indicated a role for the second heme group of cytochrome c_5 as an electron donor to AniA. Results from Chapter 5 suggest that the two domains of cytochrome c_5 are able to function independently of one another; the first domain as an electron donor to the terminal oxidase, and the second domain providing another major route of electron transfer to AniA (Figure 6.1).

As the second heme group of cytochrome c_5 is similar to third heme group of CcoP, it is possible that both of these two domains receive electrons from the second heme group of

CcoP. However, as previously discussed, the midpoint redox potentials of these heme groups and their homologues from other organisms are unknown. In the absence of this information, it is suggested that the second heme group of cytochrome c_5 receives electrons from the first heme group of the same protein, as the electron transfer distance between these two heme groups would be shorter and would therefore favour directional electron transfer (Page *et al.*, 1999). One could speculate that the midpoint potentials of the second heme group of cytochrome c_5 and the third heme group of CcoP would be slightly higher than their most likely electron donors, i.e. the first heme group of cytochrome c_5 and the second heme group of CcoP, respectively. However, since the reduction of oxygen is energetically more favourable than the reduction of nitrite, the midpoint redox potentials of these two heme groups may in fact be lower than their predicted electron donors. This would favour electron transfer pathways that terminate in more oxidizing electron acceptors.

To determine whether the first heme group of cytochrome c_5 is an electron donor to its second heme group, a mutant strain lacking the first heme group of cytochrome c_5 could be constructed. If the second heme group is reliant on electron transfer from the first heme group, the deletion of the first heme group of cytochrome c_5 should produce the same phenotype as strains JCGC850 (Δc_5::*erm*^{R}) or JCGC860 (Δc_5::*cat*), i.e. decreased oxygen and nitrite reduction rates. Although it has previously been shown that a mutant lacking the first heme group of cytochrome c_5 results in a decreased oxygen reduction rate compared to the parent strain, the rate of nitrite reduction by this strain was never measured quantitatively (Tovell, 2007).

It has been reported that cytochrome c_5 provides the majority of electron flux to AniA in the meningococcus, whereas in the gonococcus its role is shared by the third heme group of CcoP (Hopper *et al.*, 2009; Aspholm *et al.*, 2010). Interestingly, in strains of *Neisseria meningitidis* that encode a functional nitrite reductase, their rate of nitrite reduction is 2- to 3-fold lower than that of *Neisseria gonorrhoeae* (Barth *et al.*, 2009). The meningococcus lacks the third heme-binding domain of CcoP, suggesting that the presence of this extra heme group increases the ability of the organism to reduce nitrite, as seen in the gonococcus. Although the rate of nitrite reduction is lower in the meningococcus, its rate of nitric oxide reduction is 2- to 3-fold higher than the gonococcus (Barth *et al.*, 2009). This suggests that in the environment inhabited by the meningococcus the availability of nitrite might be lower, hence the loss of a functional copy of AniA in many meningococcal strains. It also indicates that the meningococcus might encounter higher concentrations of nitric oxide externally, especially since many meningococcal isolates lack a functional AniA and therefore would not produce as much NO as a by-product of their own metabolism. As *Neisseria meningitidis* lacks a cytochrome *c* peroxidase, one could speculate that the main reactive species encountered by *Neisseria gonorrhoeae* is hydrogen peroxide rather than nitric oxide (Seib *et al.*, 2004).

6.2.3 The role of cytochrome c_4 in electron transfer to AniA

Although a cytochrome c_4 deletion mutant reduced nitrite at a lower rate than the parent strain (Table 6.2), results from this work suggest that cytochrome c_4 is not a direct electron donor to AniA. As discussed previously, cytochrome c_4 contributes to electron transfer to AniA by supplying electrons to the third heme group of CcoP via the second heme group of

CcoP. The deletion of the third heme group of CcoP has no effect on the rate of nitrite reduction of a cytochrome c_4 mutant, which suggested that cytochrome c_4 might be the only electron donor to CcoP. However, if this were true the rate of nitrite reduction of strains JCGC800 (Δc_4) and AHGC101 (CcoP C368A) would be identical, which they are not (Table 6.2). The deletion of cytochrome c_4 leaves cytochrome c_5 as the only electron donor to the second heme group of CcoP, so cytochrome c_5 would be the only electron donor to oxygen via CcoO and nitrite via the third heme group of CcoP. The lack of cytochrome c_4 therefore restricts the electron flow to the terminal oxidase and to AniA due to the bifunctionalism of cytochrome c_5. The arrangement of redox components shown in Figure 6.1 would mean that, when no oxygen was available, all of the electrons that would be normally destined for the reduction of oxygen could be diverted back to AniA to allow nitrite reduction to occur. This implies that electrons from both cytochromes c_4 and c_5 could be donated to AniA at different stages in the electron transfer chain, and therefore means that, in the absence of oxygen, cytochrome c_4 can also indirectly transfer any electrons it receives to AniA via the third heme group of CcoP, thus increasing the flexibility of the electron transfer chain. Furthermore, this suggests that it would be very rare for the gonococcus to encounter high levels of oxygen, and therefore it would need to be able to quickly divert electrons to nitrite once any available oxygen had been utilised. The low level of molecular oxygen available to the gonococcus explains its requirement for the high affinity *cbb*$_3$-type terminal oxidase (which has a low K_m for oxygen), i.e. to scavenge any available oxygen so that it can be reduced preferentially over nitrite. In this way the bacterium is able to use the most efficient terminal electron acceptor at all times.

6.2.4 Further electron transfer pathways to AniA

Although the third heme group of CcoP and the second heme group of cytochrome c_5 provide the majority of electrons to AniA, the identity of the electron donor(s) responsible for the final 12% of electron transfer remains elusive. The deletion of cytochrome c_2 from strain JCGC320 (CcoP C368A c_5Δ200-279) decreased the rate of nitrite reduction by this strain to 8% of the parental rate, and this difference was statistically significant (Table 6.2). Furthermore, the deletion of cytochrome c_2 from a background lacking cytochrome c_5 always resulted in a decreased rate of nitrite reduction compared to a single mutant lacking cytochrome c_5 (Table 6.2). This suggests that cytochrome c_2 might also form an independent electron transfer pathway to AniA.

Cytochrome *c*′ and Laz were also potential electron donors to the nitrite reductase. Deletion of either of these redox proteins resulted in a decreased rate of nitrite reduction compared to the parent strain (Table 6.2). However, when genes encoding these proteins were deleted from strains JCGC320 or JCGC321 (CcoP C368A c_5Δ200-279), the resulting triple mutants still retained nitrite reduction capability (Table 6.2). This suggests that neither of these redox proteins account for the residual nitrite reduction activity of strains JCGC320 and JCGC321, and also that they do not constitute major electron pathways to AniA.

In order to determine the identity of the remaining electron transfer pathways to AniA, the construction of quadruple mutants would be required. The deletion of the *laz* or *cycA* (encoding cytochrome c_4) genes from strain JCGC350 (CcoP C368A c_5Δ200-279 Δc_2) might decrease the ability of the gonococcus to reduce nitrite. For the latter, the construction of a

double mutant strain lacking cytochrome c_4 and the second heme-binding domain of cytochrome c_5 would be required to ensure that the combination of these two mutations produce viable gonococci. Many attempts to construct this double mutant have thus far been unsuccessful. In some ways this is not surprising, since a mutant lacking the *cycA* and *cycB* genes cannot be constructed. However, the fact that the oxygen reduction rate of strain JCGC310 (c_5Δ200-279) is the same as that of the parent strain suggests that the electron flux to the terminal oxidase is unaffected by this mutation. If follows that the deletion of cytochrome c_4 from this mutant should result in the same phenotype as a single cytochrome c_4 mutation, at least in terms of oxygen reduction rates. The fact that this is not the case suggests that the second domain of cytochrome c_5 might compensate for the loss of cytochrome c_4 in strain JCGC800 (Δc_4), and therefore cannot be deleted in a background lacking cytochrome c_4. Another possibility is that the second heme group of cytochrome c_5 also donates electrons to the cytochrome *c* peroxidase, CCP, and that the absence of cytochrome c_5 renders CCP unable to quench any reactive oxygen species that might build up in a cytochrome c_4 mutant. Further attempts to construct this double mutant might be required.

6.2.5 Modelling electron transfer across the periplasm to AniA

Two factors must be considered when modelling potential electron transfer pathways to AniA. First, the redox potentials of candidate electron donors should favour electron transfer to AniA. Secondly, the redox centres of candidate electron donors and the copper centres of AniA must be sufficiently close to allow efficient electron transfer. The midpoint potentials of the two copper centres of AniA can be predicted based on values obtained from other

organisms. The midpoint potential of Type 1 copper centres (which receives electrons from other components of the electron transfer chain) range from + 247 mV to + 298 mV (Table 6.1). One would therefore expect the midpoint potential of the Type 2 copper centre (where the reduction of nitrite takes place) to be greater than that of the Type 1 copper centre. In fact the opposite is true, as the midpoint potential of Type 2 copper centres range from + 172 mV to + 244 mV (Table 6.1). It had been speculated for many years that the binding of nitrite to the Type 2 copper centre might increase its midpoint potential to a value greater than that for the Type 1 copper centre, thus allowing electron transfer to proceed from the Type 1 copper centre to the Type 2 copper centre (Olesen *et al.*, 1998; Pinho *et al.*, 2004). Indeed, recent research on the copper-containing nitrite reductase of *Alcaligenes xylosoxidans* revealed that the midpoint potential of the Type 2 copper centre increased on the binding of nitrite from + 244 mV to + 300 mV, which is greater than the midpoint potential of its Type 1 copper centre (+ 255 mV) (Leferink *et al.*, 2011). It is likely that the gonococcal nitrite reductase functions in this fashion. If so, the midpoint potentials of the second heme group of cytochrome c_5 and the third heme group of CcoP probably lie within the range of + 200 mV to + 340 mV, as is found in other *c*-type cytochromes (Table 6.1). This would be comparable to the Type 1 copper centre of AniA. One could speculate that the increase in redox potential at the Type 2 copper centre of AniA due to the binding of nitrite might provide sufficient oxidizing power to pull electrons from the inner-membrane-bound cytochromes c_5 and CcoP to the Type 1 copper centre, thus allowing electrons to reach and reduce nitrite to nitric oxide.

It is important for electron transfer processes to be efficient, as an inefficient electron

transfer chain generates higher levels of reactive oxygen species (Gray & Winkler, 2010). For efficient electron transfer to occur, the maximum distance between redox centres should not exceed 14 Å (Leys & Scrutton, 2004; Bashir *et al.*, 2010). However, it has been suggested that electron transfer can occur over distances of 20 Å by a process known as electron tunnelling (Gray & Winkler, 2003, 2010). Electron tunnelling allows electrons to pass between redox components embedded within proteins, and the tertiary structure of redox proteins allows electrons to tunnel more efficiently from one redox site to another (Gray & Winkler, 2003). For electron transfer reactions to occur over greater distances, either mobile electron carriers or the formation of supercomplexes is required. Electron transfer distances between gonococcal redox proteins can be crudely estimated (Figure 6.2). Although there are no solved structures for dimeric cytochromes c_5, the structure of the monomeric cytochrome c_5 from *Shewanella putrefaciens* has been determined (Bartalesi *et al.*, 2002). As the diameter of this protein is 30 Å, a dimeric cytochrome c_5 could have an estimated diameter of roughly 60 Å. The type 1 copper centre of AniA is located roughly 45 Å away from the outer membrane (Boulanger & Murphy, 2002), and the potential binding of cytochrome c_2 could effectively decrease the distance across the periplasm by a further 20 Å, estimated from the structure of the *Pseudoalteromonas haloplanktis* TAC125 AniA protein (Nojiri *et al.*, unpublished). The cytochrome CcoP subunit of the terminal oxidase is predicted to extend roughly 40 Å from the inner membrane into the periplasm (Buschmann *et al.*, 2010), and, assuming that the third heme-binding domain is of a similar size to the second domain of cytochrome c_5, the CcoP *C*-terminal extension could protrude a further 30 Å into the periplasm. The remaining distance between redox proteins across the periplasm could therefore be between 35 and 65 Å, assuming a periplasmic width of 170 Å (Li *et al.*, 2011). If

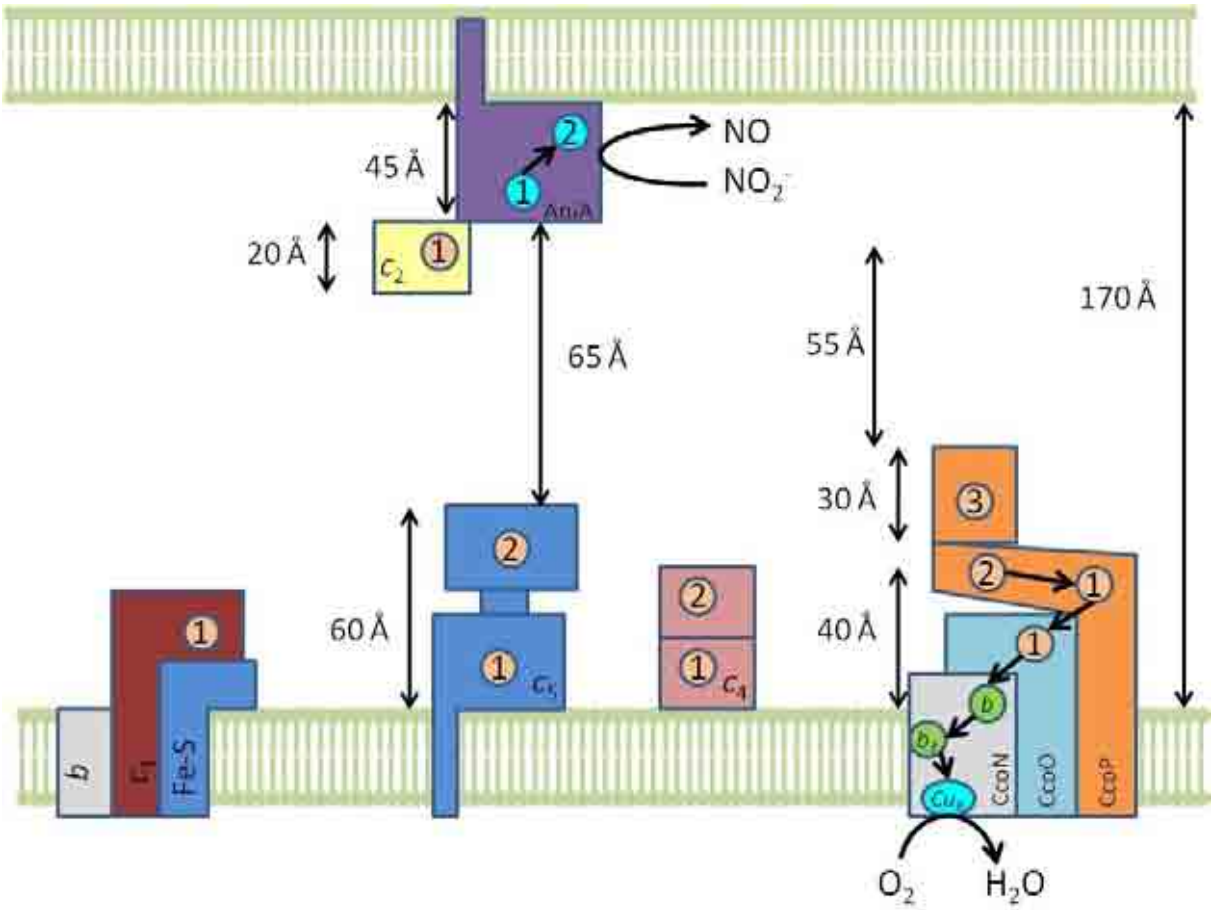


Figure 6.2 – Modelling of the distances between redox components located in both inner and outer membranes

Calculations of distances based on crystal structures obtained for similar proteins and protein complexes (Bartalesi *et al.*, 2002; Boulanger & Murphy, 2002; Buschmann *et al.*, 2010; Nojiri *et al.*, unpublished). Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity.

the predicted distances between the gonococcal redox proteins are correct, electron transfer to components such as AniA might be inefficient. The high respiratory capacity of the gonococcus could therefore prevent the build-up of reactive oxygen species that could be generated as a result of this inefficiency. Conversely, it is possible that the width of the gonococcal periplasm may be less than the predicted 170 Å. If one assumes that AniA would have to be in physical contact with its electron donors (i.e. cytochrome c_5 and CcoP) to receive electrons, one could use the predicted sizes of these redox proteins to determine the width of the gonococcal periplasm, which would give a value of between 105 Å to 115 Å. Alternatively, the formation of complexes between AniA and its inner membrane-bound electron donors might provide sufficient force to effectively pull the cytoplasmic and outer membranes together, thus decreasing the distance between redox centres and improving the efficiency of electron transfer. A further hypothesis could be that some components of the electron transfer chain might act as electron shuttles, moving electrons from redox proteins on the inner membrane to those on the outer membrane.

6.2.6 AniA cannot be reduced by ascorbate-TMPD

Ascorbate-TMPD was effective as an artificial electron donor in oxygen reduction assays, where rates of oxygen reduction by cultures were 5.1- to 6.5-fold higher than in assays when lactate was used as the electron donor (Table 3.1). However, when ascorbate-TMPD was used as an electron donor in nitrite reduction assays, the rate of nitrite reduction for all strains tested was negligible compared to assays when lactate was used as the electron donor (data not shown). TMPD mediates electron transfer from ascorbate into the electron transfer chain, and feeds electrons into the bound cytochromes *c* of the terminal oxidase

and the cytochrome bc_1 complex, as well as unbound cytochromes *c*, in this case likely including cytochromes c_4, c_5 and c_2 (Chang *et al.*, 2010). It has been reported that TMPD reduces bound cytochrome *c* more rapidly than free cytochrome *c* (Hill & Nicholls, 1980). Efficient electron transfer to the terminal oxidase is therefore manifest by the high rates of oxygen reduction observed. The midpoint potentials of the oxidized and reduced forms of ascorbate and TMPD are + 58 mV and + 260 mV, respectively. The midpoint potential of TMPD is slightly lower than the midpoint potentials of some *c*-type cytochromes described in Table 6.0. This suggests that electrons from ascorbate-TMPD might also enter the electron transfer chain at cytochromes c_1, c_2, c_4 and c_5. This could explain why the rates of oxygen reduction by various mutant strains varies, as shown in Chapter 3. It follows that electrons from ascorbate-TMPD should be available to AniA for the reduction of nitrite. However, electrons from ascorbate-TMPD pass to the terminal oxidase more rapidly than the other *c*-type cytochromes. As oxygen is a more powerful oxidizing agent than nitrite, any remaining electrons that enter via cytochromes c_4, c_5 or c_2 will preferentially pass to the terminal oxidase rather than to AniA, resulting in the poor nitrite reduction rates observed when ascorbate-TMPD is used as an electron donor rather than lactate. This hypothesis could be tested by measuring the rate of nitrite reduction with ascorbate-TMPD as an electron donor in the presence of cyanide. Cyanide binds to cytochrome b_3 of the CcoN subunit of the terminal oxidase, and therefore might prevent electrons from ascorbate-TMPD from entering at the terminal oxidase, potentially allowing the electrons supplied to other *c*-type cytochromes to reach AniA. As the midpoint potential of TMPD is higher than that of the Type 1 copper centre of AniA, this would explain why ascorbate-TMPD is a poor electron donor to AniA.

6.3 Role of the lipid-associated azurin, Laz, in the gonococcal electron transfer chain

Copper (II) ions can react with peroxide molecules to form superoxide, which can be converted into oxygen (Pires dos Santos *et al.*, 1998). As the lipid-associated azurin, Laz, contains copper and is localised to the outer membrane, it has been speculated that Laz might be involved in protection against reactive oxygen species such as peroxide. It has also been reported that a Laz deletion mutant is sensitive to copper, suggesting that Laz might be important for copper sequestration (Wu *et al.*, 2005). As described previously, manganese is used by gonococci as a non-enzymic detoxification mechanism against superoxide, hydrogen peroxide and hydroxyl radicals (Seib *et al.*, 2004). It could be speculated that the sequestration of copper ions by Laz might provide the gonococcus with a similar non-enzymic protection system against reactive oxygen species. This could explain why the gonococcal azurin, unlike those found in other bacteria, is lipid-associated and does not seem to be involved in nitrite reduction (Gotschlich & Seiff, 1987; Cannon, 1989). If the function of the gonococcal azurin has evolved to be involved solely in copper sequestration, this would explain why Laz was not found to be a major electron donor to the nitrite reductase, AniA (shown in Chapter 5).

6.4 Role of the enigmatic cytochrome c_2

It was reported in Chapter 4 that cytochrome c_2 is expressed at a very low level compared to cytochrome c_4, which suggested a minor role for the cytochrome c_2 protein. However, the rate of nitrite reduction of a mutant lacking the *cccA* gene was approximately half that of the

parent strain (Table 6.2), implicating cytochrome c_2 in electron transfer to AniA. The requirement of cytochrome c_2 for the maximal rate of nitrite reduction by the gonococcus suggests that its stoichiometry would need to be similar to that of the other *c*-type cytochromes.

There do not seem to be any bacteria outside of the *Neisseria* genus that synthesize the cytochrome c_2 homologue as a single polypeptide. However, as discussed in Chapter 4, the majority of hits from a BLAST database search for proteins similar to cytochrome c_2 are heme-binding domains fused to copper-containing nitrite reductases. This suggests that cytochrome c_2 could once have been fused to the AniA protein in the gonococcus. One could speculate that through evolution it has been translocated by homologous recombination to a separate chromosomal locus, where it is now expressed as a single unit separately from the rest of the AniA protein. There are examples of similar examples in the literature of loss of domains or subunits from families of proteins. NapA is the catalytic subunit of the periplasmic nitrate reductases, and it was believed that it always required the *c*-type cytochrome, NapB, as an electron donor (Jepson *et al.*, 2006). For example, *Escherichia coli* NapA interacts with NapB, and *Rhodobacter sphaeroides* NapA tightly binds NapB, (Arnoux *et al.*, 2003; Jepson *et al.*, 2007). One of few exceptions is *Desulfovibrio desulfuricans*, which does not encode NapB, and as a result NapA exists as a monomer in this bacterium. It has been speculated that this might allow NapA to receive electrons from multiple electron donors, such as NapM or NapG (Marietou *et al.*, 2005). The loss of cytochrome c_2 from the AniA polypeptide chain might therefore have allowed the nitrite reductase to be reduced by multiple electron donors, such as cytochromes c_5 or CcoP. However, there is a lack of DNA

sequence homology between flanking regions of cytochrome c_2 and AniA, which to a degree contradicts this hypothesis.

The precise function of cytochrome c_2 is as yet unknown. Although it could be an electron donor to AniA, its unique properties indicate an alternative role for this protein in the gonococcal electron transfer chain. Based on the results described in this work, possible roles for cytochrome c_2 are suggested in the following sub-sections.

6.4.1 Cytochrome c_2 as an electron donor to ScoI

Homologues of the ScoI protein are found in both eukaryotes and prokaryotes. In many cases, the protein has been shown to be essential for the insertion of di-copper Cu_A centres into proteins such as the cytochrome aa_3 oxidases of yeast and many prokaryotes containing this type of terminal oxidase (Nittis *et al.*, 2001; Banci *et al.*, 2011). *Bradyrhizobium japonicum* encodes an ScoI protein that is essential for the formation of its aa_3-type, but not its cbb_3-type, oxidase (Buhler *et al.*, 2010). It has been suggested that ScoI of *Thermus thermophilus* is a thiol-disulphide oxidoreductase rather than a copper chaperone, and is responsible for maintaining the correct oxidation state of the Cu_A-cysteine ligands during insertion of Cu_A into the ba_3 oxidase (Abriata *et al.*, 2008). Interestingly, the pathogenic *Neisseria* encode a ScoI protein but do not contain an aa_3-type terminal oxidase, suggesting that ScoI has a different function in these bacteria. Indeed, both *N. gonorrhoeae* and *N. meningitidis* encode a ScoI homologue that has been implicated in protection against oxidative damage to periplasmic proteins (Seib *et al.*, 2003).

Although cd_1-type nitrite reductases contain a *c*-type cytochrome domain, most NirK-like nitrite reductases contain only copper binding sites (Bertini *et al.*, 2006). However, as discussed in Chapter 4, some bacteria encode a *C*-terminal extension that contains a heme-binding domain. The presence of this additional domain often coincides with the presence of a ScoI homologue within the same operon as the copper-containing nitrite reductase, and conversely bacteria with copper-containing nitrite reductases that lack the heme-binding domain lack the ScoI homologue within the same operon (Bertini *et al.*, 2006). These authors speculated that the heme-binding domain found in these unusual NirK-like nitrite reductases is involved in electron transfer to the ScoI homologue, which could aid in the biogenesis of the copper centres of the nitrite reductase. Although ScoI homologues are generally thought to insert only Cu_A centres into proteins, there is no evidence to suggest that it could not facilitate the insertion of perhaps type 1 or type 2 copper centres into proteins such as AniA or Laz. The gonococcal ScoI homologue is not located within the same operon as AniA. However, as the gonococcal cytochrome c_2 is similar to the *C*-terminal extensions of the aforementioned unusual nitrite reductases, it is possible that it could be an electron donor to ScoI, and therefore be involved in the insertion of copper centres into AniA (Figure 6.3). Consequently, in a mutant lacking cytochrome c_2, a population of the AniA protein might lack one or both copper sites. This could explain the decreased rate of nitrite reduction by a cytochrome c_2 deletion mutant compared to the parent strain. Although Western analysis would show that the same level of AniA is present in cytochrome c_2 deletion mutants, this method does not distinguish between apo- and holo-forms of the AniA protein, the former lacking the full complement of copper sites. According to Seib *et al.*, the deletion of the *scoI* gene has no effect on the ability of the gonococcus to grow under oxygen-sufficient or

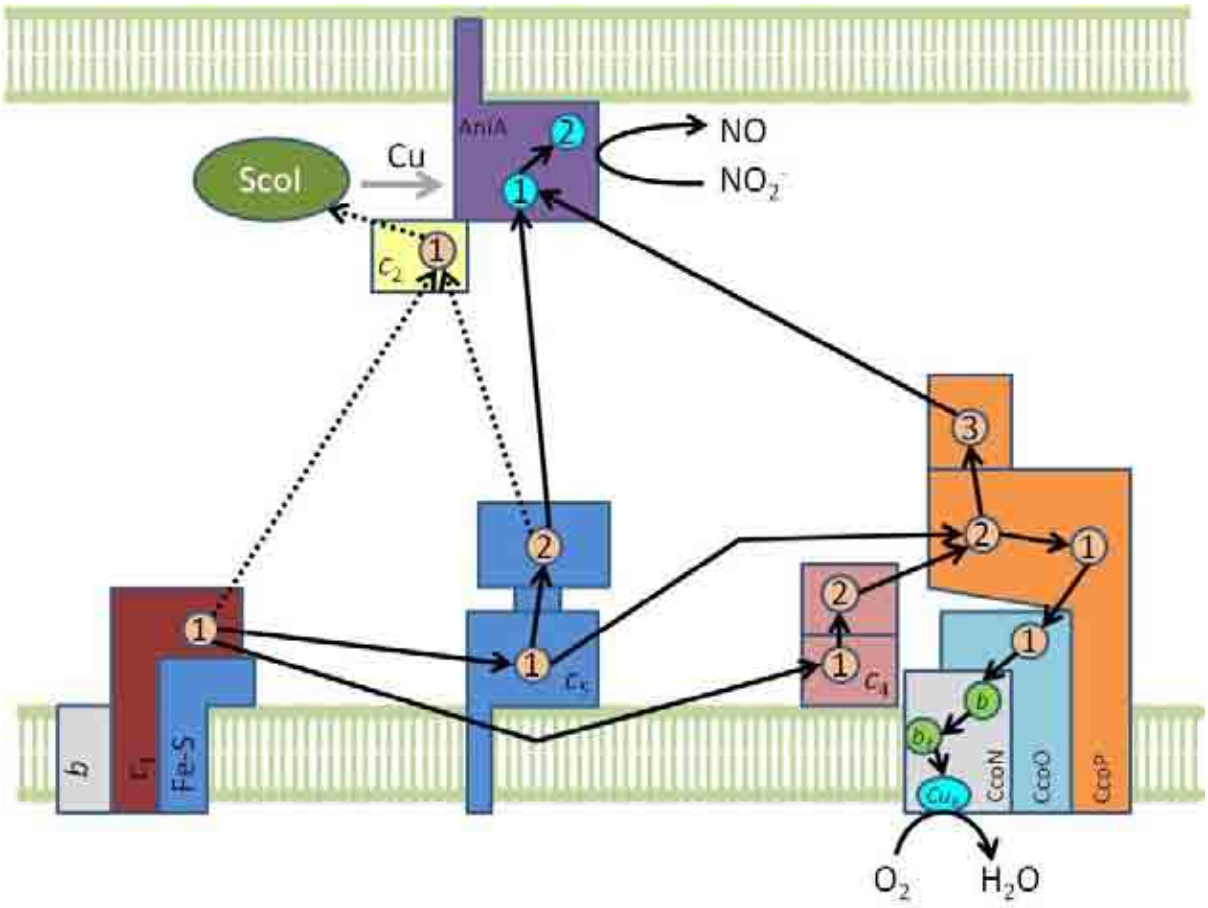


Figure 6.3 – Model of cytochrome c_2 as a possible electron donor to ScoI

Model of the electron transfer pathways to the *cbb*$_3$-type terminal oxidase and the nitrite reductase, AniA. Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways; dotted lines represent potential electron transfer routes. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity. Note that in this model cytochrome c_2 is attached to AniA, but transfers electrons to ScoI to allow the insertion of copper centres into AniA.

oxygen-limited, nitrite-supplemented conditions (Seib *et al.*, 2003). However, rates of nitrite reduction by the ScoI mutant were never determined (A. McEwan, personal communication). Consequently, it cannot be ruled out that the function of ScoI might be to aid in the insertion of copper centres into the nitrite reductase AniA, and that its absence might decrease the efficiency of copper insertion into AniA rather than preventing it entirely. Further research would be required to determine whether the above hypothesis is correct. For example, the growth of cytochrome c_2 deletion mutants under copper-replete conditions could restore its rate of nitrite reduction to that of the parent strain.

6.4.2 Cytochrome c_2 as a redox sensor

An alternative role for cytochrome c_2 in the gonococcus could be that of a redox sensor. Many heme-based sensors have been reported in the literature and can be divided into four main subfamilies: the heme-PAS domain family; the H-NOX (heme-nitric oxide/oxygen-binding) family; the CooA family; and the GCS (globin-coupled sensor) family (Gilles-Gonzalez & Gonzalez, 2005). In nearly all cases, *b*-type heme is bound to heme-based sensor proteins. The only two published exceptions are the DcrA protein from *Desulfovibrio vulgaris* Hildenborough and proteins encoded by the genes GSU0935 and GSU0582 from *Geobacter sulfurreducens*, all of which, unusually, contain *c*-type heme (Fu *et al.*, 1994; Pokkuluri *et al.*, 2008). DcrA is a novel transmembrane *c*-type cytochrome that is believed to act as a sensor of either oxygen concentration or redox potential. It is a methyl-accepting chemotaxis protein and its *C*-terminal domain is homologous to cytochromes *c*′ (Fu *et al.*, 1994). *Geobacter sulfurreducens* encodes two transmembrane proteins that each have a PAS domain within which *c*-type heme can bind (Pokkuluri *et al.*, 2008). The proteins are believed

to exist as monomers bound to the cytoplasmic membrane, where their heme group is able to sense a change in redox state of the periplasm. The detection of this stimulus causes the two proteins to undergo swapped dimerization, allowing the transduction of this signal from their periplasmic to their cytoplasmic domains (Pokkuluri *et al.*, 2008). The low accumulation of the cytochrome c_2 protein compared to the other gonococcal *c*-type cytochromes suggests a different role for this protein than simply as an electron donor to a terminal reductase. Such a low abundance of this protein implicates a role in which a high turnover of this protein or its prosthetic group would be advantageous. The ability for a redox sensor to be switched on and off rapidly allows an organism to adapt quickly to internal and external environmental changes. A fast turnover rate or low concentration of a protein would therefore decrease the amount of background noise detected by the organism and allow it to fine-tune a given response to a stimulus. Although this could mean that cytochrome c_2 might be a redox sensor, the protein has no obvious transmitter domain. It is therefore unclear how signals detected by cytochrome c_2 could be converted into a response by the bacterium. However, this does not preclude the possibility that cytochrome c_2 could interact with the transmitter domain of another protein.

6.4.3 Cytochrome c_2 as a regulator of electron flux

Although cytochrome c_2 does not appear to be a redox sensor, it is possible that it might be involved in regulating the flux of electrons to the terminal reductases. Some data in this work suggest a model in which cytochrome c_2 might somehow occlude the transfer of electrons from cytochrome c_5 to the cytochrome cbb_3 complex (Figure 6.4). The deletion of cytochrome c_2 from a cytochrome c_4 deletion mutant improved the oxygen reduction rate of

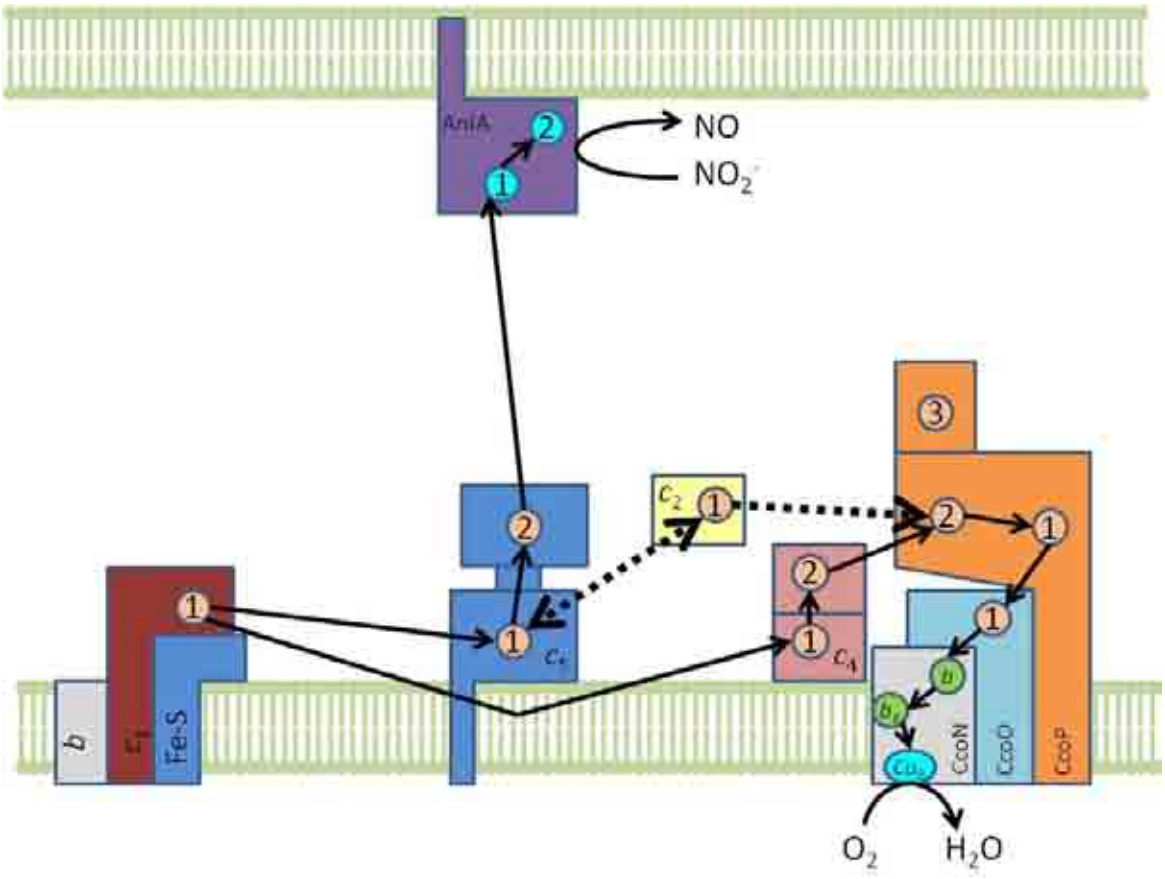


Figure 6.4 – Model of cytochrome c_2 as a possible regulator of electron flow to the terminal reductases

Model of the electron transfer pathways to the *cbb*$_3$-type terminal oxidase and the nitrite reductase, AniA. Numbers in orange circles represent heme groups, and numbers in blue circles represent copper centres. Solid lines represent probable electron transfer pathways; dotted lines represent potential electron transfer routes. The dimeric cytochrome bc_1 complex and trimeric AniA protein complexes are both shown as monomers for simplicity.

this strain compared to a single cytochrome c_4 deletion mutant (Table 3.1). This suggests that the loss of cytochrome c_2 increases electron flux through cytochrome c_5, thereby increasing electron flow to the terminal oxidase. It could therefore be speculated that cytochrome c_5, rather than cytochrome c_1 of the bc_1 complex, is an electron donor to cytochrome c_2. However, when cytochrome c_2 was deleted from a cytochrome c_5 deletion mutant, the rate of oxygen reduction was lower than that of a single cytochrome c_5 mutant (Table 3.1). The deletion of cytochrome c_2 therefore does not result in an increased flux of electrons through cytochrome c_4. There are a few possibilities to explain this phenomenon. If the electron transfer capacity of cytochrome c_4 was exceeded by the additional electron flux provided by the loss of cytochrome c_2, this could cause the quinone pool to become more reduced and potentially result in the generation of reactive oxygen species by the cytochrome bc_1 complex. The accumulation of reactive oxygen species could therefore decrease the oxygen reduction rate of the cytochrome c_2 c_5 double mutant compared to the single cytochrome c_5 mutant. However, another reason for the decreased oxygen reduction rate of the cytochrome c_2 c_5 double mutant could be that cytochrome c_2 occludes the transfer of electrons from cytochrome c_5 to the cytochrome cbb_3 complex, perhaps by competing with cytochrome c_5 for docking space at the terminal oxidase (Figure 6.4). This model suggests that in the absence of cytochrome c_5, cytochrome c_2 might be able to transfer electrons from the cytochrome bc_1 complex to the terminal oxidase. This could be an artefact of the artificial electron transfer system created by the deletion of one or more electron transfer components. It is possible that when cytochrome c_5 is deleted, its electron transfer role might be substituted by cytochrome c_2 under these unnatural conditions. Cytochrome c_4 is believed to be tightly associated with the cytochrome cbb_3 complex,

allowing cytochrome c_4 to donate electrons to the terminal oxidase. In a cytochrome c_4 deletion mutant, it is possible that cytochrome c_2 cannot compensate for the loss of cytochrome c_4 as it cannot transfer electrons to the terminal oxidase via the cytochrome c_4 binding site. This could explain the different oxygen reduction capacities of the cytochrome c_2 c_5 and c_2 c_4 deletion mutants, and suggests that cytochrome c_2 could interact with cytochrome c_5 to decrease the efficiency of electron transfer from cytochrome c_5 to the terminal oxidase.

As cytochrome c_2 might regulate electron flow from cytochrome c_5 to the cytochrome *cbb*$_3$ complex, it could therefore also regulate electron flow to AniA. If cytochrome c_2 occluded or competed with the transfer of electrons from cytochrome c_5 to its binding site on the terminal oxidase, electron flux from cytochrome c_5 to the terminal oxidase could be partially blocked by cytochrome c_2. This could result in the diversion of electrons to AniA via the second heme group of cytochrome c_5 (Figure 6.4), and would therefore allow cytochrome c_5 to act as an efficient branch point between the cytochrome *cbb*$_3$ oxidase and AniA. The low stoichiometry of cytochrome c_2 compared to cytochrome c_5 could prevent complete inhibition of electron transfer from cytochrome c_5 to the terminal oxidase, but might be at a sufficient level to allow electrons to be diverted to AniA. In this fashion, cytochrome c_2 could be acting as a regulator of electron flux in the gonococcal electron transfer chain.

The model described in Figure 6.4 might explain the inability of the Neisserial Insertional Complementation System (NICS) to complement the loss of cytochrome c_2. As the synthesis of proteins within the NICS is “leaky”, an excess of cytochrome c_2 might be synthesised, even

in the absence of IPTG. This excess amount of cytochrome c_2 might increase the competition between cytochrome c_2 and cytochrome c_5 for binding to the terminal oxidase, resulting in a greater flux of electrons to the second heme group of cytochrome c_5. In theory, this should increase the flow of electrons to AniA and therefore increase the rate of nitrite reduction of the complemented cytochrome c_2 mutant. However, the increase in competition between cytochromes c_5 and c_2 could result in a lower electron flux to the third heme group of CcoP as fewer electrons would available to CcoP from cytochrome c_5. This would decrease the electron flux to AniA via CcoP and result in a decreased nitrite reduction rate. Hence in a background containing the ectopic copy of cytochrome c_2, the resulting increase in electron flux from the second heme group of cytochrome c_5 to AniA could offset the lower electron flux from the third heme group of CcoP to AniA. Consequently, the nitrite reduction rate of a cytochrome c_2 deletion mutant would not be restored to that of the parent strain by complementation with the NICS system, due to the excess cytochrome c_2 synthesised in this strain.

6.5 Glycosylation of electron transfer chain components

Neisseria gonorrhoeae encodes a pilin O-glycosylation pathway, encoded by the *pglL* gene, which is able to glycosylate pilin subunits, PilE (Aas *et al.*, 2007). Pilin glycosylation often occurs in pathogenic bacteria, and is believed to contribute to antigenic variation in order to protect the bacterium from the host immune system (Aas *et al.*, 2007; Ku *et al.*, 2009; Nothaft & Szymanski, 2010). Although PilE is the most abundant gonococcal glycoprotein, it has been reported that PglL is able to glycosylate a further 11 proteins, of which 6 proteins are involved in electron transfer and other related redox reactions. These proteins are CcoP,

cytochrome c_5, AniA, Laz (lipid-associated azurin), ScoI and DsbA (Vik *et al.*, 2009). It is interesting to note that all of the glycosylated electron transfer components found to date are proteins that are either localised to the outer membrane, or are associated with electron transfer to these outer membrane proteins. This could suggest that ScoI and/or DsbA might be involved with AniA and Laz in some way, the former perhaps in the insertion of copper centres into AniA and Laz. It was previously hypothesised that cytochrome c_2 might have previously existed as a domain of the AniA protein. Interestingly, none of the AniA-cytochrome c_2 homologues described in Chapter 4 are glycosylated. Although AniA is glycosylated, this glycosylation plays no role in the process of nitrite reduction (Ku *et al.*, 2009). One could speculate that the advantage that the glycosylation of AniA could give the gonococcus in terms of evasion of the host immune system might have been sufficient selective pressure for cytochrome c_2 to have been lost from the AniA polypeptide chain and translocated to an alternative chromosomal locus.

6.6 The identity of electron donors to the cytochrome *c* peroxidase, CCP

The identity of electron transfer pathways to cytochrome *c* peroxidase (Ccp) have not been determined during this project. Although it is possible that the reduction of peroxide by cytochrome Ccp contributes to the generation of proton motive force and energy conservation, the major role of Ccp is more likely to be in the protection of the gonococcus from oxidative stress (Turner *et al.*, 2003; Seib *et al.*, 2004). As *Neisseria meningitidis* does not encode a cytochrome *c* peroxidase, it could be speculated that perhaps hydrogen peroxide is a more abundant reactive oxygen species in the niche of the gonococcus (Seib *et al.*, 2004).

In di-heme cytochrome *c* peroxidases, electrons are transferred from the electron transfer chain to the high potential heme group, and peroxide is reduced at the low potential heme group. The midpoint potential of the high potential heme group is often higher than that of many other *c*-type cytochromes. For example, the high potential heme groups found in the cytochrome *c* peroxidases of *Pseudomonas aeruginosa* and *Nitrosomonas europaea* (which are 48% and 46% identical to the gonococcal Ccp, respectively) have midpoint potentials of +320 and +450 (Table 6.1). This suggests that the gonococcal Ccp could receive electrons from any of the other *c*-type cytochromes or from the lipid-associate azurin, Laz. Furthermore, it is conceivable that pathways of electron transfer to cytochrome Ccp could be the same as for AniA, as both of these proteins are located on the outer membrane. Laz has been shown to provide some form of protection to the gonococcus against hydrogen peroxide (Wu *et al.*, 2005), and previous work has implicated cytochrome c_5 and Laz in electron transfer to CCP (Tovell, 2007; Li, 2009). The gonococcus has a gene encoding a catalase that has an extremely high activity (Johnson *et al.*, 1993; Johnson *et al.*, 1996). As a consequence, in order to measure the peroxidase activity of cytochrome Ccp the construction of mutant strains with a catalase-negative background would be required.

6.7 Physical structure of electron transfer chain components

To date, the only electron transfer chain component in *N. gonorrhoeae* that has had its structure determined is the nitrite reductase, AniA. Many of the other redox proteins in this bacterium have homologues that can be used to estimate their size and midpoint potentials. Nevertheless, some of the gonococcal redox proteins described in this work appear to be novel, and as a result it is difficult to fully elucidate their functions. For example, cytochrome

c_2 has only very low similarity to other *c*-type cytochromes, and its role remains unclear. If the structure of this protein could be solved it could give insight as to its function, and may explain why it is present in such low concentrations in the gonococcus. Furthermore, the determination of its midpoint potential might allow some of the previously discussed hypotheses to be either supported or discounted. Similarly, if the structure and midpoint potential of either the second heme-binding domain of cytochrome c_5 or the homologous third heme-binding domain of CcoP were determined, the interaction of these redox components with AniA might be better understood.

It is highly likely that the redox components of the electron transfer chain form supercomplexes in order to function. Supercomplexes allow for more efficient electron transfer between components, as the distance between redox centres would be reduced (Schägger, 2001). A method exists for the isolation of these supercomplexes by blue-native PAGE (Wittig *et al.*, 2006). In native PAGE proteins are not denatured and therefore complexes can remain intact. Supercomplexes are separated by electrophoresis and can be subsequently extracted from the gel. These protein complexes can then be separated by electrophoresis on an SDS-PAGE gel, which splits the supercomplexes into their constituent parts. The resulting SDS-PAGE gel can then be probed using antibodies in a Western-blot, or stained for covalently bound heme to allow the identification of proteins in each supercomplex. This method has been successfully employed in *Paracoccus denitrificans*, where cytochrome c_{552} (cycM) has been found to form complexes with Complexes I, III and IV. Although a role for cytochrome c_{552} was not determined, it was found to be unimportant for stabilising the structure of supercomplexes (Stroh *et al.*, 2004). As cytochrome c_2 does

not behave as cytochromes c_4 and c_5, it is possible that it is not directly involved in electron transfer pathways. Many of the results in this work support the hypothesis that cytochrome c_2 could form a complex with cytochrome c_5, perhaps hindering electrons from passing from cytochrome c_5 to the terminal oxidase in favour of AniA. It is also possible that cytochrome c_2 forms a complex with AniA owing to the similarity of cytochrome c_2 to the heme-binding domain of the copper-containing nitrite reductases found in some bacteria. Further work, including the isolation of supercomplexes by blue native PAGE, could determine whether either of these hypotheses are correct.

6.8 Plasticity of the gonococcal electron transfer chains

Components of the gonococcal electron transfer chain could be considered to have redundant functions. For example, either cytochrome c_4 or c_5 is sufficient for the reduction of oxygen by the terminal oxidase, and there are multiple electron transfer pathways to the nitrite reductase, AniA. Although deletion of a component of either electron transfer chain results in a decrease in efficiency, the functionality of the terminal reductases is not completely lost. This could provide the gonococcus with a selective advantage, for if one component became mutated or became a target of the host immune system or an antibiotic or chemotherapeutic agent, the bacterium might be able to continue to generate sufficient proton motive force for survival using its remaining redox components.

Although the gonococcus seems to encode functionally redundant redox proteins, the pathways of electron transfer to oxygen and nitrite can be considered to function independently of one another. If we consider the redox-active centres of the proteins as

separate moieties, the gonococcal electron transfer chains demonstrate very little obvious cross-talk. For example, the third heme group of CcoP functions independently of the other two heme groups, and the two heme groups of cytochrome c_5 have different, seemingly isolated functions. As cytochrome c_4 consists of two heme-binding domains, it is possible that it could, like cytochrome c_5, be bifunctional. It has been shown that cytochrome c_4 forms one half of the bifurcated pathway of electron transfer to the terminal oxidase, which would require at least one heme-binding domain. This potentially leaves another heme-binding domain of cytochrome c_4 available for additional electron transfer pathways, such as electron transfer to the third heme group of CcoP or to outer membrane proteins such as AniA or CCP. However, a review of cytochromes c_4 from other bacteria suggests that both domains of cytochromes c_4 are required for electron transfer to the terminal oxidase, acting as an electron wire from the cytochrome bc_1 complex to the terminal oxidase (Abergel *et al.*, 2003; Bertini *et al.*, 2006).

The oxygen tension in gonococcal biofilms is low, and the expression of many anaerobically-expressed genes is upregulated under these conditions (Falsetta *et al.*, 2009). As the depth of the biofilm increases, the level of oxygen available to the gonococcus decreases. It is therefore believed that aerobic respiration is most important during the formation of biofilms, and that the reduction of alternative terminal electron acceptors is used once biofilms are established, as oxygen would be unavailable to bacteria that are not surface-exposed (Falsetta *et al.*, 2011). The arrangement of the gonococcal electron transfer chain allows the gonococcus to adapt quickly to changes in availability of electron acceptors in the environment, without having to encode multiple, unique electron transfer components.

Instead, it uses a limited number of redox proteins to subtly change the proportion of electrons supplied to each terminal electron acceptor, allowing the gonococcus to use the most oxidizing electron acceptor available at any given time.

Bibliography

Aas FE, Vik A, Vedde J, Koomey M & Egge-Jacobsen W (2007) *Neisseria gonorrhoeae* O-linked pilin glycosylation: functional analyses define both the biosynthetic pathway and glycan structure. *Molecular Microbiology* **65**: 607-624.

Abergel C, Nitschke W, Malarte G, Bruschi M, Claverie J-M & Giudici-Orticoni M-T (2003) The structure of *Acidithiobacillus ferrooxidans* c_4-cytochrome: a model for complex-induced electron transfer tuning. *Structure* **11**: 547-555.

Abriata LA, Banci L, Bertini I, Ciofi-Baffoni S, Gkazonis P, Spyroulias GA, Vila AJ & Wang S (2008) Mechanism of Cu_A assembly. *Nature Chemical Biology* **4**: 599-601.

Ahuja U, Kjelgaard P, Schulz BL, Thöny-Meyer L & Hederstedt L (2009) Haem-delivery proteins in cytochrome *c* maturation System II. *Molecular Microbiology* **73**: 1058-1071.

Ainscough EW, Bingham AG, Brodie AM, Ellis WR, Gray HB, Loehr TM, Plowman JE, Norris GE & Baker EN (1987) Spectrochemical studies on the blue copper protein azurin from *Alcaligenes denitrificans*. *Biochemistry* **26**: 71-82.

Alderton WK, Cooper CE & Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal* **357**: 593-615.

Allen JWA, Daltrop O, Stevens JM & Ferguson SJ (2003) *C*-type cytochromes: diverse structures and biogenesis systems pose evolutionary problems. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **358**: 255-266.

Alric J, Lavergne J, Rappaport F, Vermeglio A, Matsuura K, Shimada K & Nagashima KVP (2006) Kinetic performance and energy profile in a roller coaster electron transfer chain: a study of modified tetraheme-reaction center constructs. *Journal of the American Chemical Society* **128**: 4136-4145.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403-410.

Ambler RP & Tobari J (1985) The primary structures of *Pseudomonas* Am1 Amicyanin and Pseudoazurin - 2 new sequence classes of blue copper proteins. *Biochemical Journal* **232**: 451-457.

Anon (1944) Sulphonamide-resistant gonorrhoea. *The Lancet* **244**: 693-694.

Anraku Y & Gennis RB (1987) The aerobic respiratory chain of *Escherichia coli*. *Trends in Biochemical Sciences* **12**: 262-266.

Anraku Y (1988) Bacterial electron transport chains. *Annual Review of Biochemistry* **57**: 101-132.

Archibald FS & Duong MN (1986) Superoxide-dismutase and oxygen-toxicity defenses in the genus *Neisseria*. *Infection and Immunity* **51**: 631-641.

Arciero DM & Hooper AB (1994) A di-heme cytochrome *c* peroxidase from *Nitrosomonas europaea* catalytically active in both the oxidized and half-reduced states. *Journal of Biological Chemistry* **269**: 11878-11886.

Arnoux P, Sabaty M, Alric J, Frangioni B, Guigliarelli B, Adriano JM & Pignol D (2003) Structural and redox plasticity in the heterodimeric periplasmic nitrate reductase. *Nature Structural Biology* **10**: 928-934.

Arslan E, Kannt A, Thöny-Meyer L & Hennecke H (2000) The symbiotically essential cbb_3-type oxidase of *Bradyrhizobium japonicum* is a proton pump. *FEBS Letters* **470**: 7-10.

Aspholm M, Aas FE, Harrison OB, Quinn D, Vik Å, Viburiene R, Tønjum T, Moir J, Maiden MCJ & Koomey M (2010) Structural alterations in a component of cytochrome *c* oxidase and molecular evolution of pathogenic *Neisseria* in humans. *PLoS Pathogens* **6**: e1001055.

Banci L, Bertini I, Cavallaro G & Ciofi-Baffoni S (2011) Seeking the determinants of the elusive functions of Sco proteins. *FEBS Journal* **278**: 2244-2262.

Barker PD & Ferguson SJ (1999) Still a puzzle: why is haem covalently attached in *c*-type cytochromes? *Structure* **7**: R281-R290.

Bartalesi I, Bertini I, Hajieva P, Rosato A & Vasos PR (2002) Solution structure of a monoheme ferrocytochrome *c* from *Shewanella putrefaciens* and structural analysis of sequence-similar proteins: functional implications. *Biochemistry* **41**: 5112-5119.

Barth KR, Isabella VM & Clark VL (2009) Biochemical and genomic analysis of the denitrification pathway within the genus *Neisseria*. *Microbiology-SGM* **155**: 4093-4103.

Bashir Q, Volkov AN, Ullmann GM & Ubbink M (2010) Visualization of the encounter ensemble of the transient electron transfer complex of cytochrome *c* and cytochrome *c* peroxidase. *Journal of the American Chemical Society* **132**: 241-247.

Berks BC, Ferguson SJ, Moir JWB & Richardson DJ (1995) Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochimica et Biophysica Acta-Bioenergetics* **1232**: 97-173.

Bertagna V, Erre R, Rouelle F, Chemla M, Petitdidier S & Levy D (2001) Electrochemical study for the characterisation of wet silicon oxide surfaces. *Electrochimica Acta* **47**: 129-136.

Bertini I, Cavallaro G & Rosato A (2006) Cytochrome *c*: occurrence and functions. *Chemical Reviews* **106**: 90-115.

Bertsova YV & Bogachev AV (2002) Operation of the cbb_3-type terminal oxidase in *Azotobacter vinelandii*. *Biochemistry-Moscow* **67**: 622-626.

Bevilacqua PC & Blose JM (2008) Structures, kinetics, thermodynamics, and biological functions of RNA hairpins. *Annual Review of Physical Chemistry* **59**: 79-103.

Biswas GD, Sox T, Blackman E & Sparling PF (1977) Factors affecting genetic transformation of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **129**: 983-992.

Biswas GD, Burnstein KL & Sparling PF (1986) Linearization of donor DNA during plasmid transformation in *Neisseria gonorrhoeae*. *Journal of Bacteriology* **168**: 756-761.

Bonete MJ, Martinez-Espinosa RM, Pire C, Zafrilla B & Richardson DJ (2008) Nitrogen metabolism in haloarchaea. *Saline Systems* **4**: 9.

Boulanger MJ & Murphy MEP (2002) Crystal structure of the soluble domain of the major anaerobically induced outer membrane protein (AniA) from pathogenic *Neisseria*: A new class of copper-containing nitrite reductases. *Journal of Molecular Biology* **315**: 1111-1127.

Bowyer JR & Crofts AR (1981) On the mechanism of photosynthetic electron-transfer in *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides*. *Biochimica et Biophysica Acta* **636**: 218-233.

Bueno E, Bedmar EJ, Richardson DJ & Delgado MJ (2008) Role of *Bradyrhizobium japonicum* cytochrome c_{550} in nitrite and nitrate respiration. *FEMS Microbiology Letters* **279**: 188-194.

Buhler D, Rossmann R, Landolt S, Balsiger S, Fischer HM & Hennecke H (2010) Disparate pathways for the biogenesis of cytochrome oxidases in *Bradyrhizobium japonicum*. *Journal of Biological Chemistry* **285**: 15704-15713.

Buschmann S, Warkentin E, Xie H, Langer JD, Ermler U & Michel H (2010) The structure of cbb_3 cytochrome oxidase provides insights into proton pumping. *Science* **329**: 327-330.

Cannon JG (1989) Conserved lipoproteins of pathogenic *Neisseria* species bearing the H.8 epitope - lipid-modified azurin and H.8 outer membrane protein. *Clinical Microbiology Reviews* **2**: S1-S4.

Cardinale JA & Clark VL (2000) Expression of AniA, the major anaerobically induced outer membrane protein of *Neisseria gonorrhoeae*, provides protection against killing by normal human sera. *Infection and Immunity* **68**: 4368-4369.

Cardinale JA & Clark VL (2005) Determinants of nitric oxide steady-state levels during anaerobic respiration by *Neisseria gonorrhoeae*. *Molecular Microbiology* **58**: 177-188.

Carter DC, Melis KA, Odonnell SE, Burgess BK, Furey WF, Wang BC & Stout CD (1985) Crystal-structure of *Azotobacter* cytochrome c_5 at 2.5Å resolution. *Journal of Molecular Biology* **184**: 279-295.

Catlin BW (1991) *Branhamaceae* fam. nov., a proposed family to accommodate the genera *Branhamella* and *Moraxella*. *International Journal of Systematic Bacteriology* **41**: 320-323.

Cava F, Zafra O & Berenguer J (2008) A cytochrome *c* containing nitrate reductase plays a role in electron transport for denitrification in *Thermus thermophilus* without involvement of the *bc* respiratory complex. *Molecular Microbiology* **70**: 507-518.

Ceri H, Olson ME, Stremick C, Read RR, Morck D & Buret A (1999) The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology* **37**: 1771-1776.

Chang HY, Ahn Y, Pace LA, Lin MT, Lin YH & Gennis RB (2010) The diheme cytochrome c_4 from *Vibrio cholerae* is a natural electron donor to the respiratory cbb_3 oxygen reductase. *Biochemistry* **49**: 7494-7503.

Chung GT, Yoo JS, Oh HB, Lee YS, Cha SH, Kim SJ & Yoo CK (2008) Complete genome sequence of *Neisseria gonorrhoeae* NCCP11945. *Journal of Bacteriology* **190**: 6035-6036.

Clark VL, Campbell LA, Palermo DA, Evans TM & Klimpel KW (1987) Induction and repression of outer membrane proteins by anaerobic growth of *Neisseria gonorrhoeae*. *Infection and Immunity* **55**: 1359-1364.

Clark VL, Knapp JS, Thompson S & Klimpel KW (1988) Presence of antibodies to the major anaerobically induced Gonococcal outer membrane protein in sera from patients with Gonococcal infections. *Microbial Pathogenesis* **5**: 381-390.

Covian R, Gutierrez-Cirlos EB & Trumpower BL (2004) Anti-cooperative oxidation of ubiquinol by the yeast cytochrome bc_1 complex. *Journal of Biological Chemistry* **279**: 15040-15049.

Criss AK & Seifert HS (2008) *Neisseria gonorrhoeae* suppresses the oxidative burst of human polymorphonuclear leukocytes. *Cellular Microbiology* **10**: 2257-2270.

Criss AK, Katz BZ & Seifert HS (2009) Resistance of *Neisseria gonorrhoeae* to non-oxidative killing by adherent human polymorphonuclear leucocytes. *Cellular Microbiology* **11**: 1074-1087.

Crofts AR, Shinkarev VP, Kolling DRJ & Hong SJ (2003) The modified Q-cycle explains the apparent mismatch between the kinetics of reduction of cytochromes c_1 and b_H in the bc_1 complex. *Journal of Biological Chemistry* **278**: 36191-36201.

Crofts AR (2004) The cytochrome bc_1 complex: Function in the context of structure. *Annual Review of Physiology* **66**: 689-733.

D'mello R, Purchase D, Poole RK & Hill S (1997) Expression and content of terminal oxidases in *Azotobacter vinelandii* grown with excess NH_4^+ are modulated by O_2 supply. *Microbiology-UK* **143**: 231-237.

Daldal F, Cheng S, Applebaum J, Davidson E & Prince RC (1986) Cytochrome c_2 is not essential for photosynthetic growth of *Rhodopseudomonas capsulata*. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 2012-2016.

Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 6640-6645.

Davidsen T, Rodland EA, Lagesen K, Seeberg E, Rognes T & Tonjum T (2004) Biased distribution of DNA uptake sequences towards genome maintenance genes. *Nucleic Acids Research* **32**: 1050-1058.

de Gier JWL, Schepper M, Reijnders WNM, van Dyck SJ, Slotboom DJ, Warne A, Saraste M, Krab K, Finel M, Stouthamer AH, van Spanning RJM & van der Oost J (1996) Structural and functional analysis of aa_3-type and cbb_3-type cytochrome c oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. *Molecular Microbiology* **20**: 1247-1260.

de Vries S & Schroder I (2002) Comparison between the nitric oxide reductase family and its aerobic relatives, the cytochrome oxidases. *Biochemical Society Transactions* **30**: 662-667.

Deeudom M, Rock J & Moir J (2006) Organization of the respiratory chain of *Neisseria meningitidis*. *Biochemical Society Transactions* **34**: 139-142.

Deeudom M, Koomey M & Moir JWB (2008) Roles of *c*-type cytochromes in respiration *in Neisseria meningitidis*. *Microbiology-SGM* **154**: 2857-2864.

Degli Esposti M, De Vries S, Crimi M, Ghelli A, Patarnello T & Meyer A (1993) Mitochondrial cytochrome *b* - evolution and structure of the protein. *Biochimica et Biophysica Acta* **1143**: 243-271.

Dorward DW, Garon CF & Judd RC (1989) Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **171**: 2499-2505.

Downs AJ & Jones CW (1975) Respiration-linked proton translocation in *Azotobacter vinelandii*. *FEBS Letters* **60**: 42-46.

Dunne WM (2002) Bacterial adhesion: Seen any good biofilms lately? *Clinical Microbiology Reviews* **15**: 155-166.

Edwards JL & Apicella MA (2004) The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clinical Microbiology Reviews* **17**: 965-981.

Ellfolk N, Ronnberg M, Aasa R, Andreasson LE & Vanngard T (1983) Properties and function of the 2 hemes in *Pseudomonas* cytochrome *c* peroxidase. *Biochimica et Biophysica Acta* **743**: 23-30.

Esser L, Quinn B, Li YF, Zhang MQ, Elberry M, Yu L, Yu CA & Xia D (2004) Crystallographic studies of quinol oxidation site inhibitors: a modified classification of inhibitors for the cytochrome bc_1 complex. *Journal of Molecular Biology* **341**: 281-302.

Falsetta ML, Bair TB, Ku SC, Hoven RNV, Steichen CT, McEwan AG, Jennings MP & Apicella MA (2009) Transcriptional profiling identifies the metabolic phenotype of gonococcal biofilms. *Infection and Immunity* **77**: 3522-3532.

Falsetta ML, Steichen CT, McEwan AG, Cho C, Ketterer M, Shao J, Hunt J, Jennings MP & Apicella MA (2011) The composition and metabolic phenotype of *Neisseria gonorrhoeae* biofilms. *Frontiers in Microbiology* **2**: 75.

Fee JA, Chen Y, Todaro TR, Bren KL, Patel KM, Hill MG, Gomez-Moran E, Loehr TM, Ai JY, Thony-Meyer L, Williams PA, Stura E, Sridhar V & McRee DE (2000) Integrity of *Thermus thermophilus* cytochrome c_{552} synthesized by *Escherichia coli* cells expressing the host-specific cytochrome *c* maturation genes, *ccmABCDEFGH*. Biochemical, spectral, and structural characterization of the recombinant protein. *Protein Science* **9**: 2074-2084.

Ferguson SJ, Jackson JB & McEwan AG (1987) Anaerobic respiration in the *Rhodospirillaceae* - characterization of pathways and evaluation of roles in redox balancing during photosynthesis. *FEMS Microbiology Reviews* **46**: 117-143.

Fischer RS, Martin GC, Rao P & Jensen RA (1994) *Neisseria gonorrhoeae* possesses two nicotinamide adenine dinucleotide-independent lactate dehydrogenases. *FEMS Microbiology Letters* **115**: 39-44.

Fu RD, Wall JD & Voordouw G (1994) DcrA, a *c*-type heme-containing methyl-accepting protein from *Desulfovibrio vulgaris* Hildenborough, senses the oxygen concentration or redox potential of the environment. *Journal of Bacteriology* **176**: 344-350.

García-Horsman JA, Barquera B, Rumbley J, Ma JX & Gennis RB (1994a) The superfamily of heme-copper respiratory oxidases. *Journal of Bacteriology* **176**: 5587-5600.

García-Horsman JA, Berry E, Shapleigh JP, Alben JO & Gennis RB (1994b) A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* that lacks Cu_A. *Biochemistry* **33**: 3113-3119.

Gerth K, Irschik H, Reichenbach H & Trowitzsch W (1980) Myxothiazol, an antibiotic from *Myxococcus fulvus* (*Myxobacterales*) .1. Cultivation, isolation, physicochemical and biological properties. *Journal of Antibiotics* **33**: 1474-1479.

Gilles-Gonzalez MA & Gonzalez G (2005) Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *Journal of Inorganic Biochemistry* **99**: 1-22.

Gotschlich EC & Seiff ME (1987) Identification and gene structure of an azurin-like protein with a lipoprotein signal peptide in *Neisseria gonorrhoeae*. *FEMS Microbiology Letters* **43**: 253-255.

Govezensky D, Greener T, Segal G & Zamir A (1991) Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. *Journal of Bacteriology* **173**: 6339-6346.

Gray HB & Winkler JR (2003) Electron tunneling through proteins. *Quarterly Reviews of Biophysics* **36**: 341-372.

Gray HB & Winkler JR (2010) Electron flow through metalloproteins. *Biochimica et Biophysica Acta-Bioenergetics* **1797**: 1563-1572.

Gray KA, Grooms M, Myllykallio H, Moomaw C, Slaughter C & Daldal F (1994) *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a Cu_A Center. *Biochemistry* **33**: 3120-3127.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS & Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Analytical Biochemistry* **126**: 131-138.

Greiner LL, Edwards JL, Shao J, Rabinak C, Entz D & Apicella AA (2005) Biofilm formation by *Neisseria gonorrhoeae*. *Infection and Immunity* **73**: 1964-1970.

Griess P (1879) Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt „Ueber einige Azoverbindungen". *Berichte der deutschen chemischen Gesellschaft* **12**: 426-428.

Grove J, Tanapongpipat S, Thomas G, Griffiths L, Crooke H & Cole J (1996) *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. *Molecular Microbiology* **19**: 467-481.

Guex N & Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* **18**: 2714-2723.

Haddock BA & Jones CW (1977) Bacterial respiration. *Bacteriological Reviews* **41**: 47-99.

Hägerhäll C, Magnitsky S, Sled VD, Schroder I, Gunsalus RP, Cecchini G & Ohnishi T (1999) An *Escherichia coli* mutant quinol:fumarate reductase contains an EPR-detectable semiquinone stabilized at the proximal quinone-binding site. *Journal of Biological Chemistry* **274**: 26157-26164.

Hamilton HL & Dillard JP (2006) Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. *Molecular Microbiology* **59**: 376-385.

Harte N, Silventoinen V, Quevillon E, Robinson S, Kallio K, Fustero X, Patel P, Jokinen P & Lopez R (2004) Public web-based services from the European Bioinformatics Institute. *Nucleic Acids Research* **32**: W3-W9.

Hasegawa N, Arai H & Igarashi Y (2001) Two *c*-type cytochromes, NirM and NirC, encoded in the *nir* gene cluster of *Pseudomonas aeruginosa* act as electron donors for nitrite reductase. *Biochemical and Biophysical Research Communications* **288**: 1223-1230.

Hendriks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S & Saraste M (2000) Nitric oxide reductases in bacteria. *Biochimica et Biophysica Acta-Bioenergetics* **1459**: 266-273.

Hendriks JHM, Jasaitis A, Saraste M & Verkhovsky MI (2002) Proton and electron pathways in the bacterial nitric oxide reductase. *Biochemistry* **41**: 2331-2340.

Heurlier K, Thomson MJ, Aziz N & Moir JWB (2008) The nitric oxide (NO)-sensing repressor NsrR of *Neisseria meningitidis* has a compact regulon of genes involved in NO synthesis and detoxification. *Journal of Bacteriology* **190**: 2488-2495.

Hill BC & Nicholls P (1980) Reduction and activity of cytochrome *c* in the cytochrome *c*-cytochrome aa_3 complex. *Biochemical Journal* **187**: 809-818.

Hill SA & Davies JK (2009) Pilin gene variation in *Neisseria gonorrhoeae*: reassessing the old paradigms. *FEMS Microbiology Reviews* **33**: 521-530.

Hopper A, Tovell N & Cole J (2009) A physiologically significant role in nitrite reduction of the CcoP subunit of the cytochrome oxidase cbb_3 from *Neisseria gonorrhoeae*. *FEMS Microbiology Letters* **301**: 232-240.

Horio T (1958) Terminal oxidation system in bacteria .2. Some physical and physiological properties of purified cytochromes of *Pseudomonas aeruginosa*. *Journal of Biochemistry* **45**: 267-279.

Horio T, Higashi T, Sasagawa M, Kusai K, Nakai M & Okunuki K (1960) Preparation of crystalline *Pseudomonas* cytochrome c_{551} and its general properties. *Biochemical Journal* **77**: 194-201.

Householder TC, Belli WA, Lissenden S, Cole JA & Clark VL (1999) *cis*- and *trans*-acting elements involved in regulation of *aniA*, the gene encoding the major anaerobically induced outer membrane protein in *Neisseria gonorrhoeae*. *Journal of Bacteriology* **181**: 541-551.

Householder TC, Fozo EM, Cardinale JA & Clark VL (2000) Gonococcal nitric oxide reductase is encoded by a single gene, *norB*, which is required for anaerobic growth and is induced by nitric oxide. *Infection and Immunity* **68**: 5241-5246.

Jenney FE & Daldal F (1993) A novel membrane-associated *c*-type cytochrome, cyt c_y, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. *EMBO Journal* **12**: 1283-1292.

Jepson BJN, Marietou A, Mohan S, Cole JA, Butler CS & Richardson DJ (2006) Evolution of the soluble nitrate reductase: defining the monomeric periplasmic nitrate reductase subgroup. *Biochemical Society Transactions* **34**: 122-126.

Jepson BJN, Mohan S, Clarke TA, Gates AJ, Cole JA, Butler CS, Butt JN, Hemmings AM & Richardson DJ (2007) Spectropotentiometric and structural analysis of the periplasmic nitrate reductase from *Escherichia coli*. *Journal of Biological Chemistry* **282**: 6425-6437.

Johnson SR, Steiner BM, Cruce DD, Perkins GH & Arko RJ (1993) Characterization of a catalase deficient strain of *Neisseria gonorrhoeae* - evidence for the significance of catalase in the biology of *N. gonorrhoeae*. *Infection and Immunity* **61**: 1232-1238.

Johnson SR, Steiner BM & Perkins GH (1996) Cloning and characterization of the catalase gene of *Neisseria gonorrhoeae*: Use of the gonococcus as a host organism for recombinant DNA. *Infection and Immunity* **64**: 2627-2634.

Jones CW & Redfearn ER (1967) The cytochrome system of *Azotobacter vinelandii*. *Biochimica et Biophysica Acta* **143**: 340-353.

Jones MR, Richardson DJ, McEwan AG, Ferguson SJ & Jackson JB (1990) *In vivo* redox poising of the cyclic electron-transport system of *Rhodobacter capsulatus* and the effects of the auxiliary oxidants, nitrate, nitrous oxide and trimethylamine N-oxide, as revealed by multiple short flash excitation. *Biochimica et Biophysica Acta* **1017**: 209-216.

Jurtshuk P, Aston PR & Old L (1967) Enzymatic oxidation of tetramethyl-ρ-phenylenediamine and ρ-phenylenediamine by electron transport particulate fraction of *Azotobacter vinelandii*. *Journal of Bacteriology* **93**: 1069-1078.

Jurtshuk P & Milligan TW (1974) Quantitation of tetramethyl-p-phenylenediamine oxidase reaction in *Neisseria* species. *Applied Microbiology* **28**: 1079-1081.

Jurtshuk P, Marcucci OM & McQuitty DN (1975) Tetramethyl-p-phenylenediamine oxidase reaction in *Azotobacter vinelandii*. *Applied Microbiology* **30**: 951-958.

Kadziola A & Larsen S (1997) Crystal structure of the dihaem cytochrome c_4 from *Pseudomonas stutzeri* determined at 2.2 Å resolution. *Structure* **5**: 203-216.

Kawula TH, Spinola SM, Klapper DG & Cannon JG (1987) Localization of a conserved epitope and an azurin-like domain in the H.8 protein of pathogenic *Neisseria*. *Molecular Microbiology* **1**: 179-185.

Kelly MJS, Poole RK, Yates MG & Kennedy C (1990) Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii* - mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air. *Journal of Bacteriology* **172**: 6010-6019.

Kenimer EA & Lapp DF (1978) Effects of selected inhibitors on electron transport in *Neisseria gonorrhoeae*. *Journal of Bacteriology* **134**: 537-545.

Knapp JS & Clark VL (1984) Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infection and Immunity* **46**: 176-181.

Kolonay JF, Moshiri F, Gennis RB, Kaysser TM & Maier RJ (1994) Purification and characterization of the cytochrome *bd* complex from *Azotobacter vinelandii* - comparison to the complex from *Escherichia coli*. *Journal of Bacteriology* **176**: 4177-4181.

Korszun ZR & Salemme FR (1977) Structure of cytochrome c_{555} of *Chlorobium thiosulfatophilum* - primitive low-potential cytochrome *c*. *Proceedings of the National Academy of Sciences of the United States of America* **74**: 5244-5247.

Koutny M, Kucera I, Tesarik R, Turanek J & Van Spanning RJM (1999) Pseudoazurin mediates periplasmic electron flow in a mutant strain of *Paracoccus denitrificans* lacking cytochrome c_{550}. *FEBS Letters* **448**: 157-159.

Kranz RG, Beckett CS & Goldman BS (2002) Genomic analyses of bacterial respiratory and cytochrome *c* assembly systems: *Bordetella* as a model for the System II cytochrome *c* biogenesis pathway. *Research in Microbiology* **153**: 1-6.

Kranz RG, Richard-Fogal C, Taylor JS & Frawley ER (2009) Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. *Microbiology and Molecular Biology Reviews* **73**: 510-528.

Ku SC, Schulz BL, Power PM & Jennings MP (2009) The pilin *O*-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase. *Biochemical and Biophysical Research Communications* **378**: 84-89.

Kumar P, Sannigrahi S, Scoullar J, Kahler CM & Tzeng YL (2011) Characterization of DsbD in *Neisseria meningitidis*. *Molecular Microbiology* **79**: 1557-1573.

Laratta WP, Nanaszko MJ & Shapleigh JP (2006) Electron transfer to nitrite reductase of *Rhodobacter sphaeroides* 2.4.3: examination of cytochromes c_2 and c_Y. *Microbiology-SGM* **152**: 1479-1488.

Larribe M, Taha MK, Topilko A & Marchal C (1997) Control of *Neisseria gonorrhoeae* pilin gene expression by environmental factors: Involvement of the *pilA*/*pilB* regulatory genes. *Microbiology-UK* **143**: 1757-1764.

Lazazzera BA, Beinert H, Khoroshilova N, Kennedy MC & Kiley PJ (1996) DNA binding and dimerization of the Fe-S-containing FNR protein from *Escherichia coli* are regulated by oxygen. *Journal of Biological Chemistry* **271**: 2762-2768.

Lee DJ, Bingle LEH, Heurlier K, Pallen MJ, Penn CW, Busby SJW & Hobman JL (2009) Gene doctoring: a method for recombineering in laboratory and pathogenic *Escherichia coli* strains. *BMC Microbiology* **9**.

Lee WL, Harrison RE & Grinstein S (2003) Phagocytosis by neutrophils. *Microbes and Infection* **5**: 1299-1306.

Leferink NGH, Han C, Antonyuk SV, Heyes DJ, Rigby SEJ, Hough MA, Eady RR, Scrutton NS & Hasnain SS (2011) Proton-coupled electron transfer in the catalytic cycle of *Alcaligenes xylosoxidans* copper-dependent nitrite reductase. *Biochemistry* **50**: 4121-4131.

Lewis DA (2010) The Gonococcus fights back: is this time a knock out? *Sexually Transmitted Infections* **86**: 415-421.

Leys D & Scrutton NS (2004) Electrical circuitry in biology: emerging principles from protein structure. *Current Opinion in Structural Biology* **14**: 642-647.

Li X, Parker S, Deeudom M & Moir J (2011) Tied down: tethering redox proteins to the outer membrane in *Neisseria* and other genera. *Biochemical Society Transactions* **39**: 1895-1899.

Li Y (2009) Regulation and role of electron transfer proteins in *Neisseria gonorrhoeae*. *PhD Thesis*.

Li Y, Hopper A, Overton T, Squire DJP, Cole J & Tovell N (2010) Organization of the electron transfer chain to oxygen in the obligate human pathogen *Neisseria gonorrhoeae*: roles for cytochromes c_4 and c_5, but not cytochrome c_2, in oxygen reduction. *Journal of Bacteriology* **192**: 2395-2406.

Lissenden S, Mohan S, Overton T, Regan T, Crooke H, Cardinale JA, Householder TC, Adams P, O'Conner CD, Clark VL, Smith H & Cole JA (2000) Identification of transcription activators that regulate Gonococcal adaptation from aerobic to anaerobic or oxygen-limited growth. *Molecular Microbiology* **37**: 839-855.

Mandrell RE, Griffiss JM & Macher BA (1988) Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens - carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. *Journal of Experimental Medicine* **168**: 107-126.

Mandrell RE (1992) Further antigenic similarities of *Neisseria gonorrhoeae* lipooligosaccharides and human glycosphingolipids. *Infection and Immunity* **60**: 3017-3020.

Mandrell RE & Apicella MA (1993) Lipo-oligosaccharides (LOS) of mucosal pathogens - molecular mimicry and host-modification of LOS. *Immunobiology* **187**: 382-402.

Marietou A, Richardson D, Cole J & Mohan S (2005) Nitrate reduction by *Desulfovibrio desulfuricans*: A periplasmic nitrate reductase system that lacks NapB, but includes a unique tetraheme *c*-type cytochrome, NapM. *FEMS Microbiology Letters* **248**: 217-225.

Martinkus K, Kennelly PJ, Rea T & Timkovich R (1980) Purification and properties of *Paracoccus denitrificans* azurin. *Archives of Biochemistry and Biophysics* **199**: 465-472.

Mehr IJ & Seifert HS (1998) Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Molecular Microbiology* **30**: 697-710.

Mehr IJ, Long CD, Serkin CD & Seifert HS (2000) A homologue of the recombination-dependent growth gene, *rdgC*, is involved in gonococcal pilin antigenic variation. *Genetics* **154**: 523-532.

Mehta T, Coppi MV, Childers SE & Lovley DR (2005) Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Applied and Environmental Microbiology* **71**: 8634-8641.

Mellies J, Jose J & Meyer TF (1997) The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase. *Molecular & General Genetics* **256**: 525-532.

Meyer TE, Przysiecki CT, Watkins JA, Bhattacharyya A, Simondsen RP, Cusanovich MA & Tollin G (1983) Correlation between rate-constant for reduction and redox potential as a basis for systematic investigation of reaction-mechanisms of electron-transfer proteins. *Proceedings of the National Academy of Sciences of the United States of America* **80**: 6740-6744.

Meyer TE, Cheddar G, Bartsch RG, Getzoff ED, Cusanovich MA & Tollin G (1986) Kinetics of electron-transfer between cytochromes *c*' and the semiquinones of free flavin and clostridial flavodoxin. *Biochemistry* **25**: 1383-1390.

Moir JWB & Ferguson SJ (1994) Properties of a *Paracoccus denitrificans* mutant deleted in cytochrome c_{550} indicate that a copper protein can substitute for this cytochrome in electron transport to nitrite, nitric oxide and nitrous oxide. *Microbiology-UK* **140**: 389-397.

Mooser D, Maneg O, Corvey C, Steiner T, Malatesta F, Karas M, Soulimane T & Ludwig B (2005) A four-subunit cytochrome bc_1 complex complements the respiratory chain of *Thermus thermophilus*. *Biochimica et Biophysica Acta-Bioenergetics* **1708**: 262-274.

Moran JS (2007) Gonorrhoea. *Clinical Evidence (Online)* **03**: 1-8.

Mori H & Ito K (2001) The Sec protein-translocation pathway. *Trends in Microbiology* **9**: 494-500.

Morse SA & Bartenstein L (1974) Factors affecting autolysis of *Neisseria gonorrhoeae*. *Proceedings of the Society for Experimental Biology and Medicine* **145**: 1418-1421.

Morse SA, Stein S & Hines J (1974) Glucose metabolism in *Neisseria gonorrhoeae*. *Journal of Bacteriology* **120**: 702-714.

Morse SA, Cacciapuoti AF & Lysko PG (1979) Physiology of *Neisseria gonorrhoeae*. *Advances in Microbial Physiology* **20**: 251-320.

Müller-Eberhard HJ (1986) The membrane attack complex of complement. *Annual Review of Immunology* **4**: 503-528.

Nairn CA, Cole JA, Patel PV, Parsons NJ, Fox JE & Smith H (1988) Cytidine 5'-monophospho-N-acetylneuraminic acid or a related compound is the low M_r factor from human red blood cells which induces gonococcal resistance to killing by human serum. *Journal of General Microbiology* **134**: 3295-3306.

Nassif X, Pujol C, Morand P & Eugene E (1999) Interactions of pathogenic *Neisseria* with host cells. Is it possible to assemble the puzzle? *Molecular Microbiology* **32**: 1124-1132.

Ng TCN, Laheri AN & Maier RJ (1995) Cloning, sequencing and mutagenesis of the cytochrome c_4 gene from *Azotobacter vinelandii* - characterization of the mutant

strain and a proposed new branch in the respiratory chain. *Biochimica et Biophysica Acta-Bioenergetics* **1230**: 119-129.

Nilavongse A, Brondijk THC, Overton TW, Richardson DJ, Leach ER & Cole JA (2006) The NapF protein of the *Escherichia coli* periplasmic nitrate reductase system: demonstration of a cytoplasmic location and interaction with the catalytic subunit, NapA. *Microbiology-SGM* **152**: 3227-3237.

Nittis T, George GN & Winge DR (2001) Yeast Sco1, a protein essential for cytochrome *c* oxidase function is a Cu_I-binding protein. *Journal of Biological Chemistry* **276**: 42520-42526.

Nojiri M, Koteishi H, Tsuda A, Nakagami T, Kobayashi K, Inoue T, Yamaguchi K & Suzuki S (unpublished) Electron transfer processes within and between proteins containing the HEME C and blue Cu.

Nothaft H & Szymanski CM (2010) Protein glycosylation in bacteria: sweeter than ever. *Nature Reviews Microbiology* **8**: 765-778.

Ogawa K, Sonoyama T, Takeda T, Ichiki SI, Nakamura S, Kobayashi Y, Uchiyama S, Nakasone K, Takayama SIJ, Mita H, Yamamoto Y & Sambongi Y (2007) Roles of a short connecting disulfide bond in the stability and function of psychrophilic *Shewanella violacea* cytochrome c_5. *Extremophiles* **11**: 797-807.

Oh JI & Kaplan S (2000) Redox signaling: globalization of gene expression. *EMBO Journal* **19**: 4237-4247.

Oh JI & Kaplan S (2002) Oxygen adaptation - the role of the CcoQ subunit of the cbb_3 cytochrome *c* oxidase of *Rhodobacter sphaeroides* 2.4.1. *Journal of Biological Chemistry* **277**: 16220-16228.

Olesen K, Veselov A, Zhao YW, Wang YS, Danner B, Scholes CP & Shapleigh JP (1998) Spectroscopic, kinetic, and electrochemical characterization of heterologously expressed wild-type and mutant forms of copper-containing nitrite reductase from *Rhodobacter sphaeroides* 2.4.3. *Biochemistry* **37**: 6086-6094.

Overton T, Reid EGF, Foxall R, Smith H, Busby SJW & Cole JA (2003) Transcription activation at *Escherichia coli* FNR-dependent promoters by the gonococcal FNR protein: Effects of a novel S18F substitution and comparisons with the corresponding substitution in *E. coli* FNR. *Journal of Bacteriology* **185**: 4734-4747.

Overton TW, Whitehead R, Li Y, Snyder LAS, Saunders NJ, Smith H & Cole JA (2006) Coordinated regulation of the *Neisseria gonorrhoeae* truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *Journal of Biological Chemistry* **281**: 33115-33126.

Overton TW, Justino MC, Li Y, Baptista JM, Melo AMP, Cole JA & Saraiva LM (2008) Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulfur centers. *Journal of Bacteriology* **190**: 2004-2013.

Pacher P, Beckman JS & Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews* **87**: 315-424.

Page CC, Moser CC, Chen XX & Dutton PL (1999) Natural engineering principles of electron tunnelling in biological oxidation-reduction. *Nature* **402**: 47-52.

Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, Klee SR, Morelli G, Basham D, Brown D, Chillingworth T, Davies RM, Davis P, Devlin K, Feltwell T, Hamlin N, Holroyd

S, Jagels K, Leather S, Moule S, Mungall K, Quail MA, Rajandream MA, Rutherford KM, Simmonds M, Skelton J, Whitehead S, Spratt BG & Barrell BG (2000) Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* **404**: 502-506.

Parsons NJ, Patel PV, Tan EL, Andrade JRC, Nairn CA, Goldner M, Cole JA & Smith H (1988) Cytidine 5'-monophospho-N-acetyl neuraminic acid and a low molecular weight factor from human blood cells induce lipopolysaccharide alteration in gonococci when conferring resistance to killing by human serum. *Microbial Pathogenesis* **5**: 303-309.

Parsons NJ, Andrade JRC, Patel PV, Cole JA & Smith H (1989) Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5'-monophospho-N-acetyl neuraminic acid. *Microbial Pathogenesis* **7**: 63-72.

Parsons NJ, Curry A, Fox AJ, Jones DM, Cole JA & Smith H (1992) The serum resistance of gonococci in the majority of urethral exudates is due to sialylated lipopolysaccharide seen as a surface coat. *FEMS Microbiology Letters* **90**: 295-300.

Pearson IV, Page MD, van Spanning RJM & Ferguson SJ (2003) A mutant of *Paracoccus denitrificans* with disrupted genes coding for cytochrome c_{550} and pseudoazurin establishes these two proteins as the *in vivo* electron donors to cytochrome cd_1 nitrite reductase. *Journal of Bacteriology* **185**: 6308-6315.

Pember SO, Fleck LC, Moberg WK & Walker MP (2005) Mechanistic differences in inhibition of ubiquinol cytochrome *c* reductase by the proximal Q_o-site inhibitors famoxadone and methoxyacrylate stilbene. *Archives of Biochemistry and Biophysics* **435**: 280-290.

Percival A, Corkill JE, Arya OP, Rowlands J, Alergant CD, Rees E & Annels EH (1976) Penicillinase-producing gonococci in Liverpool. *The Lancet* **308**: 1379-1382.

Pereira MM, Santana M & Teixeira M (2001) A novel scenario for the evolution of haem-copper oxygen reductases. *Biochimica et Biophysica Acta-Bioenergetics* **1505**: 185-208.

Peters A, Kulajta C, Pawlik G, Daldal F & Koch HG (2008) Stability of the cbb_3-type cytochrome oxidase requires specific CcoQ-CcoP interactions. *Journal of Bacteriology* **190**: 5576-5586.

Pettit RK & Judd RC (1992) The interaction of naturally elaborated blebs from serum-susceptible and serum-resistant strains of *Neisseria gonorrhoeae* with normal human serum. *Molecular Microbiology* **6**: 729-734.

Pinho D, Besson S, Brondino CD, de Castro B & Moura I (2004) Copper-containing nitrite reductase from *Pseudomonas chlororaphis* DSM 50135 - evidence for modulation of the rate of intramolecular electron transfer through nitrite binding to the type 2 copper center. *European Journal of Biochemistry* **271**: 2361-2369.

Pires dos Santos ML, Faljoni-Alario A, Mangrich AS & Ferreira AMD (1998) Antioxidant and pro-oxidant properties of some di-Schiff base copper(II) complexes. *Journal of Inorganic Biochemistry* **71**: 71-78.

Pitcher RS & Watmough NJ (2004) The bacterial cytochrome cbb_3 oxidases. *Biochimica et Biophysica Acta-Bioenergetics* **1655**: 388-399.

Pokkuluri PR, Pessanha M, Londer YY, Wood SJ, Duke NEC, Wilton R, Catarino T, Saigueiro CA & Schiffer M (2008) Structures and solution properties of two novel periplasmic

sensor domains with *c*-type heme from chemotaxis proteins of *Geobacter sulfurreducens*: implications for signal transduction. *Journal of Molecular Biology* **377**: 1498-1517.

Pope NR & Cole JA (1984) Pyruvate and ethanol as electron donors for nitrite reduction by *Escherichia coli* K12. *Journal of General Microbiology* **130**: 1279-1284.

Preisig O, Anthamatten D & Hennecke H (1993) Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 3309-3313.

Preisig O, Zufferey R, ThonyMeyer L, Appleby CA & Hennecke H (1996) A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *Journal of Bacteriology* **178**: 1532-1538.

Ram S, McQuillen DP, Gulati S, Elkins C, Pangburn MK & Rice PA (1998a) Binding of complement factor H to loop 5 of porin protein 1A: A molecular mechanism of serum resistance of nonsialylated *Neisseria gonorrhoeae*. *Journal of Experimental Medicine* **188**: 671-680.

Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK & Rice PA (1998b) A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. *Journal of Experimental Medicine* **187**: 743-752.

Rauhamaki V, Bloch DA, Verkhovsky MI & Wikstrom M (2009) Active site of cytochrome cbb_3. *Journal of Biological Chemistry* **284**: 11301-11308.

Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT & Lovley DR (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098-1101.

Rey L & Maier RJ (1997) Cytochrome *c* terminal oxidase pathways of *Azotobacter vinelandii*: Analysis of cytochrome c_4 and c_5 mutants and up-regulation of cytochrome *c*-dependent pathways with N_2 fixation. *Journal of Bacteriology* **179**: 7191-7196.

Richardson DJ (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiology-SGM* **146**: 551-571.

Rock JD, Mahnane MR, Anjum MF, Shaw JG, Read RC & Moir JWB (2005) The pathogen *Neisseria meningitidis* requires oxygen, but supplements growth by denitrification. Nitrite, nitric oxide and oxygen control respiratory flux at genetic and metabolic levels. *Molecular Microbiology* **58**: 800-809.

Rock JD, Thomson MJ, Read RC & Moir JWB (2007) Regulation of denitrification genes in *Neisseria meningitidis* by nitric oxide and the repressor NsrR. *Journal of Bacteriology* **189**: 1138-1144.

Rodionov DA, Dubchak IL, Arkin AP, Alm EJ & Gelfand MS (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: Comparative reconstruction of transcriptional networks. *PLoS Computational Biology* **1**: 415-431.

Sadarangani M & Pollard AJ (2010) Serogroup B meningococcal vaccines--an unfinished story. *The Lancet Infectious Diseases* **10**: 112-124.

Saraste M & Castresana J (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Letters* **341**: 1-4.

Schägger H (2001) Respiratory chain supercomplexes. *International Union of Biochemistry and Molecular Biology Life* **52**: 119-128.

Scharf B & Engelhard M (1993) Halocyanin, an archaebacterial blue copper protein (Type-I) from *Natronobacterium pharaonis*. *Biochemistry* **32**: 12894-12900.

Seib KL, Jennings MP & McEwan AG (2003) A Sco homologue plays a role in defence against oxidative stress in pathogenic *Neisseria*. *FEBS Letters* **546**: 411-415.

Seib KL, Tseng HJ, McEwan AG, Apicella MA & Jennings MP (2004) Defenses against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: distinctive systems for different lifestyles. *Journal of Infectious Diseases* **190**: 136-147.

Seib KL, Wu HJ, Kidd SP, Apicella MA, Jennings MP & McEwan AG (2006) Defenses against oxidative stress in *Neisseria gonorrhoeae*: a system tailored for a challenging environment. *Microbiology and Molecular Biology Reviews* **70**: 344-361.

Seifert HS, So M & Jeffrey HM (1991) [15] Genetic systems in pathogenic Neisseriae. In *Methods in Enzymology*. Vol. 204: Academic Press, pp. 342-357.

Shafer WM, Veal WL, Lee E, Zarantonelli L, Balthazar JT & Rouquette C (2001) Genetic organization and regulation of antimicrobial efflux systems possessed by *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Journal of Molecular Microbiology and Biotechnology* **3**: 219-224.

Shi LA, Richardson DJ, Wang ZM, Kerisit SN, Rosso KM, Zachara JM & Fredrickson JK (2009) The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* in extracellular electron transfer. *Environmental Microbiology Reports* **1**: 220-227.

Silver LE & Clark VL (1995) Construction of a translational *lacZ* fusion system to study gene regulation in *Neisseria gonorrhoeae*. *Gene* **166**: 101-104.

Simons MP, Nauseef WM & Apicella MA (2005) Interactions of *Neisseria gonorrhoeae* with adherent polymorphonuclear leukocytes. *Infection and Immunity* **73**: 1971-1977.

Smith H, Cole JA & Parsons NJ (1992) The sialylation of gonococcal lipopolysaccharide by host factors - a major impact on pathogenicity. *FEMS Microbiology Letters* **100**: 287-292.

Smith H, Yates EA, Cole JA & Parsons NJ (2001) Lactate stimulation of Gonococcal metabolism in media containing glucose: Mechanism, impact on pathogenicity, and wider implications for other pathogens. *Infection and Immunity* **69**: 6565-6572.

Snyder C & Trumpower BL (1998) Mechanism of ubiquinol oxidation by the cytochrome bc_1 complex: pre-steady-state kinetics of cytochrome bc_1 complexes containing site-directed mutants of the Rieske iron-sulfur protein. *Biochimica et Biophysica Acta-Bioenergetics* **1365**: 125-134.

Snyder CH, Merbitz-Zahradnik T, Link TA & Trumpower BL (1999) Role of the Rieske iron-sulfur protein midpoint potential in the protonmotive Q-cycle mechanism of the cytochrome bc_1 complex. *Journal of Bioenergetics and Biomembranes* **31**: 235-242.

Soulimane T, vonWalter M, Hof P, Than ME, Huber R & Buse G (1997) Cytochrome c_{552} from *Thermus thermophilus*: a functional and crystallographic investigation. *Biochemical and Biophysical Research Communications* **237**: 572-576.

Stefanelli P, Colotti G, Neri A, Salucci ML, Miccoli R, Di Leandro L & Ippoliti R (2008) Molecular characterization of nitrite reductase gene (*aniA*) and gene product in *Neisseria meningitidis* isolates: Is *aniA* essential for meningococcal survival? *International Union of Biochemistry and Molecular Biology Life* **60**: 629-636.

Steichen CT, Shao JQ, Ketterer MR & Apicella MA (2008) Gonococcal cervicitis: a role for biofilm in pathogenesis. *Journal of Infectious Diseases* **198**: 1856-1861.

Steichen CT, Cho C, Shao JQ & Apicella MA (2011) The *Neisseria gonorrhoeae* biofilm matrix contains DNA, and an endogenous nuclease controls its incorporation. *Infection and Immunity* **79**: 1504-1511.

Stern A, Brown M, Nickel P & Meyer TF (1986) Opacity genes in *Neisseria gonorrhoeae* - control of phase and antigenic variation. *Cell* **47**: 61-71.

Stevanin TM, Moir JWB & Read RC (2005) Nitric oxide detoxification systems enhance survival of *Neisseria meningitidis* in human macrophages and in nasopharyngeal mucosa. *Infection and Immunity* **73**: 3322-3329.

Stohl EA, Criss AK & Seifert HS (2005) The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Molecular Microbiology* **58**: 520-532.

Storz G & Imlay JA (1999) Oxidative stress. *Current Opinion in Microbiology* **2**: 188-194.

Strange JC & Judd RC (1994) Identification of heme containing proteins of *Neisseria gonorrhoeae*: p. 417-423 *In* C. J. Conde-Glez, S. Morse, P. Rice, F. Sparling and E. Calderón (ed.), Pathology and immunology of *Neisseriaceae*. Instituto Nacional de Salud Pública Press, Morclos, Mexico.

.

Stroh A, Anderka O, Pfeiffer K, Yagi T, Finel M, Ludwig B & Schagger H (2004) Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*. *Journal of Biological Chemistry* **279**: 5000-5007.

Sutton VR, Mettert EL, Beinert H & Kiley PJ (2004a) Kinetic analysis of the oxidative conversion of the $[4Fe\text{-}4S]^{2+}$ cluster of FNR to a $[2Fe\text{-}2S]^{2+}$ cluster. *Journal of Bacteriology* **186**: 8018-8025.

Sutton VR, Stubna A, Patschkowski T, Munck E, Beinert H & Kiley PJ (2004b) Superoxide destroys the $[2Fe\text{-}2S]^{2+}$ cluster of FNR from *Escherichia coli*. *Biochemistry* **43**: 791-798.

Taniguchi VT, Sailasutascott N, Anson FC & Gray HB (1980) Thermodynamics of metalloprotein electron-transfer reactions. *Pure and Applied Chemistry* **52**: 2275-2281.

Thomas PE, Ryan D & Levin W (1976) An improved staining procedure for detection of peroxidase activity of cytochrome P450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry* **75**: 168-176.

Thöny-Meyer L, Stax D & Hennecke H (1989) An unusual gene cluster for the cytochrome bc_1 complex in *Bradyrhizobium japonicum* and its requirement for effective root nodule symbiosis. *Cell* **57**: 683-697.

Thöny-Meyer L, Beck C, Preisig O & Hennecke H (1994) The *ccoNOPQ* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. *Molecular Microbiology* **14**: 705-716.

Tissieres A (1956) Purification, some properties and the specific biological activity of cytochrome c_4 and c_5 from *Azotobacter vinelandii*. *Biochemical Journal* **64**: 582-589.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou LX, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weldman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS,

Weidman JM, Fujii C, Borodovsky M, Karp PD, Smith HO, Fraser CM & Venter JC (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539-547.

Tovell N (2007) The role and regulation of the *c*-type cytochrome complement of *Neisseria gonorrhoeae*. *PhD Thesis*.

Trees DL & Spinola SM (1990) Localization of and immune response to the lipid-modified azurin of the pathogenic *Neisseria*. *Journal of Infectious Diseases* **161**: 336-339.

Tremblay PL, Summers ZM, Glaven RH, Nevin KP, Zengler K, Barrett CL, Qiu Y, Palsson BO & Lovley DR (2011) A *c*-type cytochrome and a transcriptional regulator responsible for enhanced extracellular electron transfer in *Geobacter sulfurreducens* revealed by adaptive evolution. *Environmental Microbiology* **13**: 13-23.

Trumpower BL (1990) Cytochrome bc_1 complexes of microorganisms. *Microbiological Reviews* **54**: 101-129.

Tseng HJ, Srikhanta Y, McEwan AG & Jennings MP (2001) Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. *Molecular Microbiology* **40**: 1175-1186.

Tsukahara Y, Morisaki T, Horita Y, Torisu M & Tanaka M (1998) Expression of inducible nitric oxide synthase in circulating neutrophils of the systemic inflammatory response syndrome and septic patients. *World Journal of Surgery* **22**: 771-777.

Turina P, Samoray D & Graber P (2003) H^+/ATP ratio of proton transport-coupled ATP synthesis and hydrolysis catalysed by CF_0F_1-liposomes. *EMBO Journal* **22**: 418-426.

Turner S, Reid E, Smith H & Cole J (2003) A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a Gram-negative bacterium. *Biochemical Journal* **373**: 865-873.

Turner SM, Moir JWB, Griffiths L, Overton TW, Smith H & Cole JA (2005) Mutational and biochemical analysis of cytochrome *c*', a nitric oxide-binding lipoprotein important for adaptation of *Neisseria gonorrhoeae* to oxygen-limited growth. *Biochemical Journal* **388**: 545-553.

Unden G & Bongaerts J (1997) Alternative respiratory pathways of *Escherichia coli*: Energetics and transcriptional regulation in response to electron acceptors. *Biochimica et Biophysica Acta-Bioenergetics* **1320**: 217-234.

Van Der Oost J, Deboer APN, Degier JWL, Zumft WG, Stouthamer AH & Vanspanning RJM (1994) The heme-copper oxidase family consists of 3 distinct types of terminal oxidases and is related to nitric oxide reductase. *FEMS Microbiology Letters* **121**: 1-9.

Vik A, Aas FE, Anonsen JH, Bilsborough S, Schneider A, Egge-Jacobsen W & Koomey M (2009) Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 4447-4452.

Virji M (2009) Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nature Reviews Microbiology* **7**: 274-286.

Visser JM, deJong GAH, deVries S, Robertson LA & Kuenen JG (1997) cbb_3-type cytochrome oxidase in the obligately chemolithoautotrophic *Thiobacillus sp* W5. *FEMS Microbiology Letters* **147**: 127-132.

Von Jagow G, Ljungdahl PO, Graf P, Ohnishi T & Trumpower BL (1984) An inhibitor of mitochondrial respiration which binds to cytochrome *b* and displaces quinone from the iron-sulfur protein of the cytochrome bc_1 complex. *Journal of Biological Chemistry* **259**: 6318-6326.

Wang W, Kinkel T, Martens-Habbena W, Stahl DA, Fang FC & Hansen EJ (2011) The *Moraxella catarrhalis* nitric oxide reductase is essential for nitric oxide detoxification. *Journal of Bacteriology* **193**: 2804-2813.

Weis W (1975) Ascorbic acid and electron transport. *Annals of the New York Academy of Sciences* **258**: 190-200.

Welsch JA & Ram S (2008) Factor H and Neisserial pathogenesis. *Vaccine* **26**: I40-I45.

Whitehead RN, Overton TW, Snyder LAS, McGowan SJ, Smith H, Cole JA & Saunders NJ (2007) The small FNR regulon of *Neisseria gonorrhoeae*: comparison with the larger *Escherichia coli* FNR regulon and interaction with the NarQ-NarP regulon. *BMC Genomics* **8**.

Wilson TGG & Wilson PW (1955) The terminal oxidation system of *Azotobacter vinelandii*. *Journal of Bacteriology* **70**: 30-34.

Winter DB & Morse SA (1975) Physiology and metabolism of pathogenic *Neisseria* - partial characterization of respiratory chain of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **123**: 631-636.

Wittig I, Braun HP & Schagger H (2006) Blue native PAGE. *Nature Protocols* **1**: 418-428.

Wood PM (1983) Why do *c*-type cytochromes exist? *FEBS Letters* **164**: 223-226.

Woods JP, Dempsey JF, Kawula TH, Barritt DS & Cannon JG (1989) Characterization of the Neisserial lipid-modified azurin bearing the H.8 epitope. *Molecular Microbiology* **3**: 583-591.

Wu HJ, Seib KL, Edwards JL, Apicella MA, McEwan AG & Jennings MP (2005) Azurin of pathogenic *Neisseria spp*. is involved in defense against hydrogen peroxide and survival within cervical epithelial cells. *Infection and Immunity* **73**: 8444-8448.

Yip ES, Burnside DM & Cianciotto NP (2011) Cytochrome c_4 is required for siderophore expression by *Legionella pneumophila*, whereas cytochromes c_1 and c_5 promote intracellular infection. *Microbiology-SGM* **157**: 868-878.

Yu EKC & Devoe IW (1980) Terminal branching of the respiratory electron transport chain in *Neisseria meningitidis*. *Journal of Bacteriology* **142**: 879-887.

Zufferey R, Preisig O, Hennecke H & ThonyMeyer L (1996) Assembly and function of the cytochrome cbb_3 oxidase subunits in *Bradyrhizobium japonicum*. *Journal of Biological Chemistry* **271**: 9114-9119.

Zuker M (1989) On finding all suboptimal foldings of an RNA molecule. *Science* **244**: 48-52.

Zumft WG, Gotzmann DJ & Kroneck PMH (1987) Type-1, blue copper proteins constitute a respiratory nitrite-reducing system in *Pseudomonas aureofaciens*. *European Journal of Biochemistry* **168**: 301-307.

Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61**: 533-616.