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
Acknowledgements

I would first like to thank Professor Jeff Cole for all of his help and support throughout my PhD. I very much appreciate his continued willingness to spend time explaining tricky concepts and sharing his vast knowledge. I cannot thank you enough for giving me so many opportunities to both begin and continue a career in research.

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The past four years of blood, sweat and tears (all literally!) have gone into my PhD, and it has been a huge journey. Thanks again to everyone who believed in me and got me through it.
## Table of Contents

### Chapter 1 Introduction .................................................................1

1.1 Introduction ................................................................................. 2
1.2 Pathogenicity and evasion of host defence mechanisms .......... 3
1.3 Oxidative and nitrosative stress protection mechanisms ......... 6
1.4 Oxidative phosphorylation ......................................................... 9
1.5 Cytochrome $cbb_3$ oxidases as members of the heme-copper oxidase family 16
1.6 Denitrification processes in the *Neisseria* genus ..................... 18
1.7 Electron transfer chains to nitrite ............................................... 23
1.8 $c$-type cytochromes as redox carriers ...................................... 25
1.9 The eight $c$-type cytochromes of *Neisseria gonorrhoeae* ............ 29
1.10 Previous characterisation of the electron transfer chain of *Neisseria gonorrhoeae* ......................................................... 35
1.11 Aims of this project ................................................................. 39

### Chapter 2 Materials and Methods .............................................41

2.1 Bacterial strains ................................................................. 42
2.2 Media and media supplements ................................................. 42
2.3 Growth of *Neisseria gonorrhoeae* ........................................ 42
2.4 Harvesting of cultures .......................................................... 46
2.5 Gram’s staining ................................................................. 46
2.6 Oxygen reduction assays ......................................................... 47
2.7 Spot testing ......................................................................... 48
2.8 Whole cell nitrite reduction assays ........................................ 48
2.9 Agarose gel electrophoresis ................................................... 51
2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoreses (SDS-PAGE) .......................... 52
2.11 Staining of gels for covalently bound heme .......................... 53
2.12 Analysis of AniA and 3xFLAG tag in bacteria by Western analysis ................................. 53
2.13 Molecular techniques ......................................................... 54
2.14 Extraction of genomic DNA from *Neisseria gonorrhoeae* .......... 59
2.15 Use of *Escherichia coli* strains for cloning experiments ......... 59
2.16 Transformation of *Neisseria gonorrhoeae*: construction of single, double and triple mutants .......... 63
2.17 Construction of plasmids for ectopic complementation of *cycA* and *cycB* mutations ................................................................. 64
2.18 Construction of a plasmid for overexpression of cytochrome $c_2$ ......................................................... 68
2.19 Site-directed mutagenesis of the potential stem loop structure upstream of the $cccA$ coding sequence ......................................................... 68
2.20 Construction of a gonococcal cytochrome $c_2$ deletion mutation conferring chloramphenicol resistance ................................................................. 70
2.21 Construction of a gonococcal cytochrome $c_5$ deletion mutation conferring chloramphenicol resistance ................................................................. 71
Chapter 3 Electron transfer to oxygen.......................................................................... 78

3.1 Introduction........................................................................................................... 79
3.2 Rates of oxygen reduction and myxothiazol inhibition by single mutants
    defective in cytochromes c₂, c₄, and c₅ ................................................................. 81
3.3 Complementation of the cycA and cycB mutations by ectopic expression of
    IPTG-inducible cycA and cycB genes ................................................................... 84
3.4 Phenotypes of double mutants defective in cytochromes c₂ and c₄ or
    cytochromes c₂ and c₅ ......................................................................................... 86
3.5 Failure to isolate a double mutant defective in both cytochromes c₄ and c₅ .... 92
3.6 IPTG-dependent survival of a double mutant defective in both cytochromes
    c₄ and c₅ complemented by an inducible copy of the ectopically located
    cycB gene ............................................................................................................. 93
3.7 Discussion ............................................................................................................ 95
   3.7.1 Essential roles of either cytochrome c₄ or cytochrome c₅ in electron
         transfer between the gonococcal cytochrome bc₁ complex and
         cytochrome oxidase ......................................................................................... 95
   3.7.2 Lack of a significant role of cytochrome c₂ in electron transfer to
         cytochrome oxidase ....................................................................................... 98
   3.7.3 Inefficient inhibition of respiration by myxothiazol .................................... 99
   3.7.4 Oxygen sensitivity during growth of single and double mutants
         defective in cytochromes c₂, c₄, or c₅ ............................................................ 101
   3.7.5 Why is a pathogen with a microaerobic lifestyle such a prolific source
         of electron transfer components? .................................................................. 102

Chapter 4 The enigmatic cytochrome c₂ ................................................................. 104

4.1 Introduction........................................................................................................... 105
4.2 Bioinformatic analysis of the gonococcal cccA gene ........................................ 105
4.3 Nitrite supplements oxygen-limited growth of gonococci .............................. 112
4.4 Cytochrome c₂ as a potential electron donor to AniA ..................................... 112
4.5 Overexpression of cytochrome c₂ ................................................................. 118
4.6 Detection of cytochrome c₂ synthesis ............................................................ 126
4.7 Regulation of cytochrome c₂ expression .......................................................... 128
4.8 Discussion ........................................................................................................... 132
   4.8.1 The low abundance of cytochrome c₂ ..................................................... 136
   4.8.2 The expression of cytochrome c₂ does not appear to be induced by
         nitrite ................................................................................................................. 137
4.8.3 The overexpression of cytochrome $c_2$ cannot complement a cytochrome $c_2$ deletion mutant ......................................................... 137
4.8.4 A novel role for cytochrome $c_2$? ............................................................. 138

Chapter 5 Electron donors to the nitrite reductase, AniA ........................................ 140

5.1 Introduction ......................................................................................................... 141
5.2 Cytochromes $c_4$ and $c_5$ as potential electron donors to AniA ..................... 142
5.3 The novel third heme group of cytochrome CcoP ........................................ 143
5.4 Site-directed mutagenesis of the third heme-binding domain of cytochrome CcoP ......................................................................................... 146
5.5 Effects of substitutions in the $C$-terminal heme-binding domain of CcoP on aerobic growth and respiration ................................................................. 148
5.6 Effects of substitutions in the $C$-terminal heme-binding domain of CcoP on oxygen-limited growth and nitrite reduction ........................................ 152
5.7 Attempted transfer of the CcoP C368A mutation into a single mutant defective in cytochrome $c_5$ ............................................................... 152
5.8 Transfer of the cytochrome $c_4$ deletion mutation into the CcoP C368A mutant .................................................................................. 160
5.9 Transfer of the cytochrome $c_2$ deletion mutation into a CcoP C368A Delta$c_4$ double mutant ................................................................. 162
5.10 Addition of mutations to the CcoP C368A Delta$c_5$ Delta200-279 double mutant ......................................................................................... 166
5.11 Cytochrome $c'_5$ as a potential electron donor to AniA .................................. 168
5.12 The lipid-associated azurin, Laz, as a potential electron donor to AniA .......... 169
5.13 Role of cytochrome $c_2$ in electron transfer to AniA ......................................... 175
5.14 Discussion ........................................................................................................ 177
5.14.1 The third heme group of CcoP as an electron donor to AniA .................... 177
5.14.2 The bifunctionality of cytochrome $c_5$ .......................................................... 178
5.14.3 Cytochrome $c_4$ as an electron donor to AniA .............................................. 179
5.14.4 Role of cytochrome $c_4$ in the electron transfer chain to nitrite .......... 182
5.14.5 Poor growth of the triple mutant lacking cytochromes $c_4$, $c_2$ and the third heme group of CcoP ............................................................. 184
5.14.6 Cytochrome $c'_5$ and the lipid-associated azurin are not major electron donors to AniA .............................................................................. 186

Chapter 6 Discussion ................................................................................................ 188

6.1 The electron transfer chain to oxygen ............................................................ 189
6.2 The electron transfer chain to nitrite ............................................................... 195
6.2.1 The third heme group of CcoP is a major electron donor to AniA .............. 195
6.2.2 The second heme group of cytochrome $c_5$ is a major electron donor to AniA ......................................................................................... 197
6.2.3 The role of cytochrome $c_4$ in electron transfer to AniA ............................ 199
6.2.4 Further electron transfer pathways to AniA ............................................. 201
6.2.5 Modelling electron transfer across the periplasm to AniA ....................... 202
6.2.6 AniA cannot be reduced by ascorbate-TMPD ........................................ 206
6.3 Role of the lipid-associated azurin, Laz, in the gonococcal electron transfer chain ............................................................................................ 208
6.4 Role of the enigmatic cytochrome c₂................................................................. 208
  6.4.1 Cytochrome c₂ as an electron donor to ScoI............................................. 210
  6.4.2 Cytochrome c₂ as a redox sensor.............................................................. 213
  6.4.3 Cytochrome c₂ as a regulator of electron flux ......................................... 214
6.5 Glycosylation of electron transfer chain components................................. 218
6.6 The identity of electron donors to the cytochrome c peroxidase, CCP........... 219
6.7 Physical structure of electron transfer chain components............................ 220
6.8 Plasticity of the gonococcal electron transfer chains .................................... 222

Bibliography.............................................................................................................. 225
List of Figures

Figure 1.1 – Electron donors to ubiquinone in Neisseria gonorrhoeae ........................................ 10
Figure 1.2 – Structure of the cytochrome \( bc_1 \) complex from chicken mitochondria............ 13
Figure 1.3 – Schematic of a typical bacterial electron transfer chain.............................. 15
Figure 1.4 – Structure of the cytochrome \( \text{cbb}_3 \)-type oxidase from Pseudomonas stutzeri................................................................................................................. 19
Figure 1.5 – Structure of hemes \( c \), \( a \), \( b \) and \( o \).............................................................. 26
Figure 1.6 – Cytochrome \( c \) biogenesis.................................................................................. 28
Figure 1.7 – Multiple alignment of cytochromes \( c_4 \) ............................................................ 31
Figure 1.8 – Multiple alignment of cytochromes \( c_5 \) .......................................................... 33
Figure 1.9 – Multiple alignment of cytochromes \( c_5 \) domain 1........................................... 34
Figure 1.10 – Pairwise alignment of cytochrome \( c_2 \) from Neisseria gonorrhoeae FA1090 and cytochrome \( c_{552} \) from Thermus thermophilus......................................................... 36
Figure 1.11 – Multiple alignment of Neisseria gonorrhoeae FA1090 cytochrome \( c_2 \) and C-termini of some copper-containing nitrite reductases......................................................... 37
Figure 2.1 – Mechanism of nitrite detection using Griess Reagents.................................... 49
Figure 3.1 – SDS-PAGE gel stained for the presence of covalently bound heme in strains F62 and JCGC860 \( (\Delta c_5::\text{cat}) \) ...................................................................... 85
Figure 3.2 – Oxygen-sufficient growth of complemented cytochrome \( c_4 \) and \( c_5 \) deletion mutant strains....................................................................................................................... 87
Figure 3.3 – SDS-PAGE gels stained for covalently bound heme in complemented cytochrome \( c_4 \) and \( c_5 \) deletion mutant strains .............................................................. 89
Figure 3.4 – Rates of oxygen reduction by washed bacterial suspensions of complemented cytochrome \( c_4 \) and \( c_5 \) deletion mutant strains.................................................. 90
Figure 3.5 – Oxygen-sufficient growth of strains F62 and JCGC880 \( (\Delta c_5 \Delta c_4 c_5I) \) in the absence and presence of IPTG..................................................................................... 94
Figure 3.6 – Scheme for electron transfer from NADH to oxygen in Neisseria gonorrhoeae .................................................................................................................................................................................. 97
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................................................................. 100
Figure 4.1 – Pairwise alignment of the amino acid sequence of AniA proteins from Moraxella catarrhalis and Neisseria gonorrhoeae .................................................................................................................. 106
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 ...................................................................................... 108
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 ...................................................................................................................... 109
Figure 4.4 – Trimeric forms of AniA proteins from Pseudoalteromonas haloplanktis TAC125 and Neisseria gonorrhoeae................................................................................................. 111
Figure 4.5 – Superimposition of monomeric forms of AniA from *Pseudoalteromonas haloplanktis* TAC125 (shown in blue) and *Neisseria gonorrhoeae* (shown in purple) ........................................................................................................... 113

Figure 4.6 – Oxygen-limited growth of strain F62 supplemented with or without sodium nitrite......................................................................................................................... 114

Figure 4.7 – Oxygen-limited growth and nitrite reduction during growth of strains F62 and JCGC851 (Δc₂), supplemented with or without nitrite.................................................. 116

Figure 4.8 – Rates of nitrite reduction and accumulation of AniA protein in strains F62 and JCGC851 (Δc₂) ........................................................................................................ 117

Figure 4.9 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc₂) and JCGC140 (c₂l) ...................................................................................................... 119

Figure 4.10 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc₂) and JCGC140 (c₂l) ......................................................................... 121

Figure 4.11 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc₂) and JCGC142 (c₂l Δc₂) ....................................................................................... 123

Figure 4.12 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc₂) and JCGC142 (c₂l Δc₂) ................................................................. 124

Figure 4.13 – Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC851 (Δc₂) and JCGC142 (c₂l Δc₂) ................................................................. 125

Figure 4.14 – Oxygen-limited nitrite-supplemented growth and accumulation of 3xFLAG-tagged proteins in cultures of strains F62, JCGC851 (Δc₂), JCGC807 (c₂ 3XFLAG) and JCGC805 (c₄ 3XFLAG) ........................................................................ 127

Figure 4.15 – Oxygen-limited growth, nitrite reduction during growth, and accumulation of 3xFLAG-tagged cytochrome c₂ protein in strains F62, JCGC851 (Δc₂) and JCGC807 (c₂ 3XFLAG) ..................................................................................... 129

Figure 4.16 – Diagram showing the potential stem loop structure that could form upstream of the translation start site of the *cccA* gene .................................................. 131

Figure 4.17 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc₂), JCGC152 (c₂l TTC Δc₂) and JCGC153 (c₂l AAG Δc₂) ......................................................... 133

Figure 4.18 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc₂), JCGC152 (c₂l TTC Δc₂) and JCGC153 (c₂l AAG Δc₂) ............... 135

Figure 5.1 – Rates of nitrite reduction by and washed bacterial suspensions of strains F62, JCGC800 (Δc₄) and JCGC850 (Δc₅) ........................................................................ 144

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 ........................................................................ 145

Figure 5.3 – Pairwise alignment of the gonococcal CcoP/CycB amino acid sequences... 147

Figure 5.4 – SDS-PAGE gel stained for covalently bound heme in CcoP third heme group mutants .................................................................................................................. 149

Figure 5.5 – Aerobic growth and rates of oxygen reduction by CcoP third heme group mutants.................................................................................................................. 150

Figure 5.6 – Oxygen-limited, nitrite-supplemented growth of CcoP third heme group mutants ................................................................................................................ 153
List of Tables

Table 2.1 – Bacterial strains used in this work .......................................................... 43
Table 2.2 – Standard protocol for PCR reactions ..................................................... 55
Table 2.3 – Primers used in this work ..................................................................... 56
Table 2.4 – Plasmids used in this work ................................................................. 60
Table 2.5 – *Neisseria gonorrhoeae* strains constructed in this work ..................... 65
Table 3.1 – Rates of oxygen reduction by washed bacterial suspensions of strains F62, JGC800 (Δc₄), JGC850 (Δc₃), JGC851 (Δc₂), JGC853 (Δc₂ Δc₄) and JGC852 (Δc₂ Δc₅) ........................................................................................................ 82
Table 5.1 – Rates of nitrite reduction by various mutants in combination with a *laz* deletion mutation .......................................................... 173
Table 5.2 – Rates of nitrite reduction by various mutants in combination with a cytochrome c₂ mutation .......................................................... 176
Table 6.1 – Midpoint potentials of redox components from various organisms .......... 192
Table 6.2 – Summary table of nitrite reduction rates measured in Chapter 5 .......... 196
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Ascorbate-TMPD</td>
<td>Ascorbate, N,N,N',N'-tetramethyl-ρ-phenylenediamine</td>
</tr>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>cycA</td>
<td>gene encoding cytochrome ( c_4 )</td>
</tr>
<tr>
<td>cycB</td>
<td>gene encoding cytochrome ( c_5 )</td>
</tr>
<tr>
<td>cccA</td>
<td>gene encoding cytochrome ( c_2 )</td>
</tr>
<tr>
<td>Fe-S</td>
<td>non-heme iron-sulphur</td>
</tr>
<tr>
<td>GCA</td>
<td>Gonococcal Agar</td>
</tr>
<tr>
<td>GCB</td>
<td>Gonococcal Broth</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl ( β )-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>Polymerase Chain Reaction</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-( β )-D-galactopyranoside</td>
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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*

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 gluconeogenesis 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 $\text{H}^+$/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 \textit{aa}_3-type oxidases; type B, \textit{ba}_3-type oxidases; or type C, \textit{cbb}_3-type oxidases (Pereira \textit{et al.}, 2001). The \textit{cbb}_3-type oxidases are the most divergent members of the heme-copper oxidase (HCO) family (Pitcher & Watmough, 2004).

The first \textit{cbb}_3-type oxidase was discovered in \textit{Bradyrhizobium japonicum} (Thöny-Meyer \textit{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\textsubscript{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 \textit{cbb}_3-type oxidase (encoded by the \textit{fixNOPQ} gene cluster) that has a very low K\textsubscript{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 \textit{et al.}, 1993; Preisig \textit{et al.}, 1996). Cytochrome \textit{cbb}_3 oxidases are often found in bacteria that inhabit microaerobic environments. As a result, many human pathogens such as \textit{Helicobacter pylori} and members of the \textit{Neisseria} genus encode a \textit{cbb}_3-type oxidase (Tomb \textit{et al.}, 1997; Parkhill \textit{et al.}, 2000).

Cytochrome \textit{cbb}_3 oxidases are composed of three subunits. The \textit{ccoN} gene encodes the catalytic subunit I of the oxidase, where oxygen is reduced to water. Subunits II and III are \textit{c}-type cytochromes encoded by the genes \textit{ccoO} and \textit{ccoP}, respectively (Saraste & Castresana,
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 *d₁* 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 \textit{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 \textit{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 \textit{aniA} promoter. In this fashion the expression of \textit{aniA} is increased. When levels of nitric oxide rise above a certain level, FNR becomes deactivated and \textit{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
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 derepressed 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, $a$-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 ccmABCDEFGH 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).
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\textsubscript{1} from the bc\textsubscript{1} complex (NG2031), and CcoO (NG1373) and CcoP (NG1371) from the cytochrome cbb\textsubscript{3} complex. A further two c-type cytochromes are outer membrane proteins, namely cytochrome c peroxidase (NG1769) and cytochrome c\textsuperscript{'} (NG1080), both of which have been previously discussed. The remaining three cytochromes, cytochromes c\textsubscript{2}, c\textsubscript{4} and c\textsubscript{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\textsubscript{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.
The gonococcal cytochrome c₄ (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 c₄ might not be attached to the cytoplasmic membrane. The role of cytochrome c₄ in other bacteria is usually as an electron donor. For example, the A. vinelandii cytochrome c₄, which is 36% identical to the cytochrome c₄ of Neisseria gonorrhoeae (Figure 1.7), exists as part of a bifurcated pathway between the cytochrome bc₁ complex and the cbb₃-type oxidase (Ng et al., 1995). The gonococcal cytochrome c₄ is 99.5% identical to the cytochrome c₄ of Neisseria meningitidis and 33% identical to the cytochrome c₄ of Vibrio cholerae (Figure 1.7). In the case of V. cholerae, cytochrome c₄ is an electron donor to the cbb₃-type oxidase (Chang et al., 2010). There has been a report that the cytochrome c₄ of Legionella pneumophila, which is 34% identical to the gonococcal cytochrome c₄ (Figure 1.7), is essential for siderophore expression (Yip et al., 2011). Therefore, although most cytochromes c₄ seem to be electron donors to cbb₃-type oxidases, this might not always be their sole function.

Gonococcal cytochrome c₅ (NG1328) is a 29 kDa protein made up of 279 residues. Residues 16-32 are mostly hydrophobic, suggesting that the gonococcal cytochrome c₅ could be tethered to the cytoplasmic membrane by a transmembrane domain. The nature of cytochromes c₅ depends on the class of proteobacteria from which they originate. Species in most classes of proteobacteria have monohemic cytochromes c₅; however, species that fall in the β-proteobacteria class, such as Neisseria gonorrhoeae, generally have dihemic cytochromes c₅. The gonococcal cytochrome c₅ is therefore most similar to cytochromes c₅.
Figure 1.7 – Multiple alignment of cytochromes c₄

Multiple alignment of cytochromes c₄ 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 monohemicytochromes $c_5$ 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 monohemicytochrome $c_5$ and dihemicytochrome $c_4$ 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 monohemicytochrome, 16.5 kDa protein of 155 residues
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**Figure 1.8 – Multiple alignment of cytochromes c₅**

Multiple alignment of cytochromes c₅ from *Neisseria gonorrhoeae* FA1090 (labelled Ng), *Chromobacterium violaceum* ATCC12472 (labelled Cv), *Thiobacillus denitrificans* 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 c₅ domain 1.

Multiple alignment of cytochromes c₅ 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 c₉ from other organisms, as it is only 26% similar in amino acid sequence to cytochromes c₂ from *Rhodobacter sphaeroides*, *Rhodopseudomonas globiformis* and *Hydrogenobacter thermophilus* c₅₅₂. Its closest relative is cytochrome c₂ from *Thermus thermophilus*, a member of the phylum Deinococcus thermos, which has been shown to transfer electrons from the bc₁ complex to both caa₃ and ba₃ oxidases (Soulimane et al., 1997; Mooser et al., 2005). Residues 19 to 141 of gonococcal cytochrome c₂ are 51% similar to residues 101-209 of *T. thermophilus* cytochrome c₅₅₂, although the cytochromes are only 28% similar overall (Figure 1.10). Although the meningococcal cytochrome c₉ is 93% identical to the gonococcal cytochrome c₂, outside of the *Neisseria* genus gonococcal cytochrome c₂ is up to 50% identical to part of some copper-containing nitrite reductases (Figure 1.11). This suggests that cytochrome c₂ 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
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| Ng   | 1 --------------------------------------------------MNTTRLPTA 9 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
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| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
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| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |
| Ng   | 10 FILCLCAAASADNSIMTKGKVYSENFIACCHKKGEGRGTAFFPLFRS 59 |
| Tt   | 51 vvdskgitrgqweevvenmkvmglqltpeeeevllylatylgrnppp-- 98 |

**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       GKRKYESSCFAQDSNNGQGLPGAFPPPLAKSDFLKND-KDAISAVIHGLEGPIKVNGKEYNSVMPAQL 434  
Mcat       GKAHYDSRCAADQDPDKGKVPNAFPPPLANSYLNADHARASSIVANGLSGKTVNGQYSVMPAIAL 465  
Psp        GESVYASCGAACHQPGTPGVPNAFPPPLAKSDYLNDPRNAIADAHNGLSGKTVNGQYVSMPAVNL 473  
Kkin       GEDYKAKLCHVEGKQEVAFPPPLAESYLNADYKVRGQFAQVKGGLSGETVNGQKFKNSVMPAVAL 477  
Ngon       GQKVYESNCIACH4KKGEGGRTSAPPFLRSDY1MNKPHLLHSMVKGINTIKVNGKTYNGFPATAI 460  

*: *   *** .*  **** .*:   . :*:.* .*:  . :*:.* .:*  . :*:.* .:*  . :*:.* .:*  

Phalo      NDEDIANVTFNLNNWDNAGGKVSAEQVAPQVRK------------------------- 467  
Bbac       SDEDAANVLTYIYSWGNKSVPGKHKTPADVKAARAGKK--------------------- 471  
Mcat       SDQQIANVITYLNSFGNKGGEIADDVAKAKKNQALILKFN------------------ 508  
Psp        DDEKTANVYILNSKKGKEITPDVAARK----------------------------- 505  
Kkin       NDEDAANVLTFVNLNSFGNKGEVKPKPEEIAAARK---------------------- 510  
Ngon       SDADIAAVATYIMNADFNGGSVTEKDVQGKKNQTDKMPSETGNGASDGIQIKPF 518  

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 $N. \text{gonorrhoeae}$ and $N. \text{meningitidis}$ 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 $\text{Myxococcus fulvus}$ 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-$\rho$-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
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 *bc₁* 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 *c₄* and *c₅* are both essential for electron transfer to the terminal oxidase, and whether cytochrome *c₂* 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 *c₄*, *c₅*, and *c₂* 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 NaHCO₃; 18 g of glucose; 2 g of L-glutamate; 0.004 g of co-carboxylase; and 0.1 g of Fe(NO₃)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 K₂HPO₄; 1 g of KH₂PO₄; 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

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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$_{650\text{ nm}}$ 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 $\text{OD}_{650\text{ nm}}$ 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 $\text{K}_2\text{HPO}_4$ and 1.13 g $\text{KH}_2\text{PO}_4$) 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 $\mu$L of the same phosphate buffer and the optical density of cells calculated by measuring the $\text{OD}_{650\text{ nm}}$ of a 100-fold dilution of the cell solution. For both oxygen and nitrite reduction assays, washed whole cells with an $\text{OD}_{650\text{ nm}}$ of between 20 and 40 were used. The quantity of cells was then calculated using the estimation of an $\text{OD}_{650\text{ nm}}$ 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$_{650}$ 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:

$$\text{Rate} = \frac{\text{gradient calculated by Oxygraph} \times \text{total vol. (μL)}}{\text{OD}_{650} \text{ nm cells} \times 0.4 \times \text{vol. cells (μL)}}$$
where \[ \text{gradient} = \frac{\Delta O_2 \text{ concentration (μM)}}{\Delta \text{ time (min)}} \]

- total vol. = total volume of chamber = 2000 μL
- \( OD_{650 \text{ nm}} \) cells = \( OD_{650 \text{ nm}} \) of 1/100 dilution of cells x 100
- 0.4 = dry weight of cells for a culture with \( OD_{650 \text{ nm}} \) 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 NaNO₂ 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:

\[
\text{Rate} = \frac{\text{gradient} \times 30 \times 20 \times \text{total vol. (μL)}}{\text{OD}_{540 \text{ nm}} \times \text{standard} \times \text{OD}_{650 \text{ nm}} \times \text{cells} \times \text{vol. cells (μL)} \times 0.4}
\]

where

- gradient = \(\frac{\Delta \text{OD}_{540 \text{ nm}}}{\Delta \text{time (s)}}\)
- 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)
- \(\text{OD}_{540 \text{ nm}} \times \text{standard}\) = OD540 nm of nitrite standard sample
- \(\text{OD}_{650 \text{ nm}} \times \text{cells}\) = OD650 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 \(\text{OD}_{650 \text{ nm}}\) 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 $T_m = 64.9 + (G\% + C\%) \times 0.41 - \frac{600}{n}$, 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

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</tr>
<tr>
<td>Step 2</td>
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<tr>
<td></td>
<td></td>
<td>T&lt;sub&gt;m&lt;/sub&gt;°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>X min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(where T&lt;sub&gt;m&lt;/sub&gt; = melting temperature of homology region)</td>
<td>(where X = Kb size of fragment to be amplified)</td>
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<tr>
<td>Step 3</td>
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**Table 2.3 – Primers used in this work**

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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

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<tr>
<th>Plasmid</th>
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<tr>
<td>pSUB11</td>
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<td>pAH131</td>
<td>ermC gene ligated into the AgeI site of pAH130</td>
<td>This work</td>
</tr>
<tr>
<td>pAH132</td>
<td>cat gene ligated into the AgeI site of pAH130</td>
<td>This work</td>
</tr>
<tr>
<td>pAH140</td>
<td>laZ gene interrupted by cat gene in pGEM® T-Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pAHC2FCD</td>
<td>336 bp of downstream DNA of the cccA gene ligated between the Xhol site and the Nhel site of pDOC-F</td>
<td>This work</td>
</tr>
<tr>
<td>pAH130</td>
<td>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</td>
<td>This work</td>
</tr>
<tr>
<td>pAHC2F</td>
<td>Source of 3xFLAG tag</td>
<td>(Lee et al., 2009)</td>
</tr>
<tr>
<td>pGCC4</td>
<td>Neisserial Insertional Complementation System ermC, Kan&lt;sup&gt;®&lt;/sup&gt;, lctP, aspC, lacI&lt;sup&gt;Q&lt;/sup&gt;, lacP</td>
<td>(Mehr &amp; Seifert, 1998)</td>
</tr>
<tr>
<td>pGCC4_cycA</td>
<td>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</td>
<td>(Mehr et al., 2000)</td>
</tr>
<tr>
<td>pGCC4_cycB</td>
<td>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</td>
<td>(Stohl et al., 2005)</td>
</tr>
</tbody>
</table>

60
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source</th>
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<tr>
<td>pGCC4_cccA</td>
<td>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</td>
<td>This work</td>
</tr>
<tr>
<td>pGCC4_cccAb</td>
<td>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</td>
<td>This work</td>
</tr>
<tr>
<td>pGCC4_cccA_loop</td>
<td>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</td>
<td>This work</td>
</tr>
<tr>
<td>pNT100</td>
<td>Gonococcal cycA gene cloned into pGEM&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pNT101</td>
<td>ermC gene flanked by AgeI sites in pGEM&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pNT102</td>
<td>ermC gene ligated into the AgeI site of pNT100</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pNT502</td>
<td>laz gene interrupted by kan&lt;sup&gt;R&lt;/sup&gt; gene in pGEM&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>Laboratory stocks</td>
</tr>
<tr>
<td>pYL4</td>
<td>kan&lt;sup&gt;R&lt;/sup&gt; gene flanked by AgeI sites in pGEM&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pYL6</td>
<td>584 bp of DNA upstream of cycB linked by an AgeI site to 448 bp of DNA downstream of cycB in pGEM&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pYL7</td>
<td>ermC gene ligated into the AgeI site of pYL6</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pYL12</td>
<td>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&lt;sup&gt;T&lt;/sup&gt;-Easy</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pYL13</td>
<td>kan&lt;sup&gt;R&lt;/sup&gt; gene ligated into the AgeI site of pYL12</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>pYL14</td>
<td>515 bp of DNA upstream of aniA linked by a BamHI site to 516 bp of DNA downstream of aniA in pGEM&lt;sup&gt;T&lt;/sup&gt;-easy</td>
<td>(Li, 2009)</td>
</tr>
<tr>
<td>pYL15</td>
<td>kan&lt;sup&gt;R&lt;/sup&gt; gene ligated into the BamHI site of pYL14</td>
<td>(Li, 2009)</td>
</tr>
</tbody>
</table>
minutes and resuspended in 400 μL of a solution of 0.1 M CaCl₂ 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 MgCl₂, 10 mM MgSO₄ 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 MgCl₂, 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 c₄ and c₅ deletion mutants, ectopically expressed copies of the cytochrome c₄ and c₅ 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

<table>
<thead>
<tr>
<th>Strain constructed</th>
<th>Recipient strain</th>
<th>Mutation added</th>
<th>DNA template*</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHGC100</td>
<td>F62</td>
<td><em>kan</em></td>
<td>pAH110</td>
<td>AHccoP_ds_F</td>
</tr>
<tr>
<td>AHGC101</td>
<td>F62</td>
<td>ccoP C368A <em>kan</em></td>
<td>pAH111</td>
<td>AHccoP_ds_R2a</td>
</tr>
<tr>
<td>AHGC102</td>
<td>F62</td>
<td>ccoP C371A <em>kan</em></td>
<td>pAH112</td>
<td>AHccoP_ds_R2a</td>
</tr>
<tr>
<td>AHGC103</td>
<td>F62</td>
<td>ccoP C371A H372A <em>kan</em></td>
<td>pAH113</td>
<td>AHccoP_ds_R2a</td>
</tr>
<tr>
<td>AHGC104</td>
<td>F62</td>
<td>ccoP C368A C371A H372A <em>kan</em></td>
<td>pAH114</td>
<td>AHccoP_ds_R2a</td>
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<tr>
<td>JGC110</td>
<td>F62</td>
<td>Δ<em>aspC-lctP</em>:placl-cycA&lt;sup&gt;+&lt;/sup&gt;*ermC</td>
<td>pGCC4_cycA</td>
<td>pGCC4_F</td>
</tr>
<tr>
<td>JGC11</td>
<td>F62</td>
<td>Δ<em>aspC-lctP</em>:placl-cycB&lt;sup&gt;+&lt;/sup&gt;*ermC</td>
<td>pGCC4_cycB</td>
<td>pGCC4_R</td>
</tr>
<tr>
<td>JGC140</td>
<td>F62</td>
<td>Δ<em>aspC-lctP</em>:placl-cccA&lt;sup&gt;+&lt;/sup&gt;50 bp upstream, ermcC</td>
<td>pGCC4_cccA_loop</td>
<td>pGCC4_R</td>
</tr>
<tr>
<td>JGC142</td>
<td>JGC140 (ΔaspC-lctP::placl-cccA&lt;sup&gt;+&lt;/sup&gt; 14 bp upstream ermcC)</td>
<td>Δ<em>aspC-lctP</em>:placl-cccA&lt;sup&gt;+&lt;/sup&gt;50 bp upstream, ermcC</td>
<td>pGCC4_cccA</td>
<td>YLC552AUF</td>
</tr>
<tr>
<td>JGC150</td>
<td>F62</td>
<td>Δ<em>aspC-lctP</em>:placl-cccA&lt;sup&gt;+&lt;/sup&gt;50 bp upstream, ermcC</td>
<td>pGCC4_cccA</td>
<td>YLC552AUF</td>
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<tr>
<td>JGC151</td>
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<td>Δ<em>aspC-lctP</em>:placl-cccA&lt;sup&gt;+&lt;/sup&gt;50 bp upstream cccA T-22A T-21A C-20G, ermcC</td>
<td>pGCC4_cccA</td>
<td>YLC552AUF</td>
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<tr>
<td>JGC152</td>
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<td>cccA::kan&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>YLC552AUF</td>
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<td>JGC153</td>
<td>JGC151 (ΔaspC-lctP::placl-cccA&lt;sup&gt;+&lt;/sup&gt; 50 bp upstream cccA T-22A T-21A C-20G, ermcC)</td>
<td>cccA::kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pYL13</td>
<td>YLC552AUF</td>
</tr>
<tr>
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<td>JGC214</td>
<td>ST_CycPFP3</td>
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<td>pAH131</td>
<td>ST_CycPRP1</td>
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<td>pAH132</td>
<td>AHC5-2int_F</td>
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<td>cccA::cat</td>
<td>pAH130</td>
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</tr>
<tr>
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<td>pAH131</td>
<td>AHC5-2int_F</td>
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<td>pAH132</td>
<td>AHC5-2int_R</td>
</tr>
<tr>
<td>JGC330</td>
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<td>pAH130</td>
<td>AHC5-2int_F</td>
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<td>pAH130</td>
<td>AHC5-2int_R</td>
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<tr>
<td>JGC340</td>
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<td>cycA::ermC</td>
<td>pNT102</td>
<td>NTACYC4 FOR</td>
</tr>
<tr>
<td>JGC350</td>
<td>JGC320 (ccoP C368A kan&lt;sup&gt;+&lt;/sup&gt; cycB Δ 200-279::ermC)</td>
<td>cccA::cat</td>
<td>pAH130</td>
<td>NTACYC4 REV</td>
</tr>
<tr>
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<td>cycA::ermC</td>
<td>pNT102</td>
<td>YLC552AUF</td>
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</tbody>
</table>

65
<table>
<thead>
<tr>
<th>Strain constructed</th>
<th>Recipient strain</th>
<th>Mutation added</th>
<th>DNA template*</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>JCGC352</td>
<td>JCGC320 (ccoP C368A kan&lt;sup&gt;*&lt;/sup&gt;)&lt;br&gt;cycB Δ200-279::ermC</td>
<td>laz::cat</td>
<td>pAH140</td>
<td>YLC552CDR</td>
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<tr>
<td></td>
<td>JCGC355 (ccoP C368A kan&lt;sup&gt;*&lt;/sup&gt;)&lt;br&gt;cycP::ermC</td>
<td>cycB Δ200-279::cat</td>
<td>pAH132</td>
<td>NTAZUFOR, AHC5-2int_F, AHC5-2int_R</td>
</tr>
<tr>
<td></td>
<td>F62</td>
<td>cccA::3xFLAG kan&lt;sup&gt;*&lt;/sup&gt;</td>
<td>pAH2F</td>
<td>AHC2AUFecoRI, AHC2CDRHel</td>
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<tr>
<td>JCGC820</td>
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<td>cycA::kan&lt;sup&gt;*&lt;/sup&gt;</td>
<td>JCGC801</td>
<td>NTCYC4 FOR, NTCYC4 REV</td>
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<tr>
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<td>cccA::cat</td>
<td>pAH107</td>
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<td>pAH105</td>
<td>YLCSAUF, YLC5CDR</td>
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<tr>
<td></td>
<td>JCGC870 (cytB::cat ΔaspC-lctp::plac-cycB&lt;sup&gt;*&lt;/sup&gt; ermC)</td>
<td>cycA::kan&lt;sup&gt;*&lt;/sup&gt;</td>
<td>JCGC801</td>
<td>NTCYC4 FOR, NTCYC4 REV</td>
</tr>
<tr>
<td></td>
<td>F62</td>
<td>laz::cat</td>
<td>pAH140</td>
<td>NTAZUFOR, NTAZUREV</td>
</tr>
</tbody>
</table>

<sup>*</sup>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 c2 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 c₅ 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*<sup>R</sup> gene. The PCR fragment containing the *cat* gene was digested with *AgeI*, then ligated into the pNT502 vector lacking the *kan*<sup>R</sup> 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 \( \text{c}_5 \)

Crossover PCR was used to construct a truncated version of cytochrome \( \text{c}_5 \). 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 \( \text{cycB} \) gene and 595 bp of coding DNA of the \( \text{cycB} \) gene. Primer AHC5-2ndBURb incorporated a stop codon, to allow the translation of the truncated version of the cytochrome \( \text{c}_5 \) protein. Primers AHC5-2ndDDF and AHC5-2ndCDR were used to amplify 612 bp of non-coding DNA downstream of the \( \text{cycB} \) 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 \( \text{AgeI} \) 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 \( \text{AgeI} \) 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\(^{\text{®}}\) T-Easy vector. The \( \text{ermC} \) gene was amplified from JCGC100 using the primers NTERY FOR and NTERY REV, which introduced \( \text{AgeI} \) sites at each end. The \( \text{cat} \) gene was amplified from pLES940 using the primers AHCatAgeIF and AHCatAgeIR, which introduced \( \text{AgeI} \) sites at each end. The \( \text{ermC} \) gene fragment, \( \text{cat} \) gene
fragment and pAH130 were digested with *Agel*, then either the *ermC* gene or the *cat* gene was ligated into the *Agel* 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 c₂

To create a FLAG-tagged version of cytochrome c₂, 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 AHC2CDRNhel. 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.northwestern.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 cbb₃ 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 cbb₃ oxidase from Bradyrhizobium japonicum has a very low Kᵣ for oxygen (Kᵣ = 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 c₁ component of the cytochrome bc₁ complex; and three genes, cccA, cycA, and cycB, encoding cytochromes of unknown function that have been designated cytochromes c₂, c₄, and c₅, 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 c₄ and c₅ (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 $\text{cycA}$ mutant that lacks cytochrome $c_4$ was 16% lower than that of the parent, and the corresponding rate for the $\text{cycB}$ 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 $\text{cccA}$ 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 $\text{cbb}_3$ 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
Table 3.1 – Rates of oxygen reduction by washed bacterial suspensions of strains F62, JCGC800 (Δc₄), JCGC850 (Δc₅), JCGC851 (Δc₂), JCGC853 (Δc₂ Δc₄) and JCGC852 (Δc₂ Δc₅)

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 \( \mu \)M 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 \( \mu \)M 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 \( \mu \)M myxothiazol still only partially inhibited gonococcal respiration. Although 1 \( \mu \)M 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 *cyc*A and *cyc*B mutations by ectopic expression of IPTG-inducible *cyc*A and *cyc*B 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 *cyc*A and *cyc*B 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 *cyc*A or *cyc*B were integrated between the *lctP* and *aspC* genes on the chromosomes of parental strain F62 and the *cyc*A or *cyc*B 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 *cyc*B gene had been deleted (Figure 3.1).

Strains JCGC10 (*c*$_4$I, 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 (Δc₅::cat)

Cultures were grown under oxygen-sufficient conditions and samples were taken after 6 hours of growth.
alternative chromosomal locus), JCGC801 (Δc₄) and JCGC820 (c₄Δc₄) 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 c₄ or cytochrome c₅ 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 c₄ and c₅ 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 c₂ and c₄ or cytochromes c₂ and c₅

The results presented above implicated both cytochromes c₄ and c₅ in electron transfer between the cytochrome bc₁ complex and cytochrome cbb₃ oxidase but suggested that cytochrome c₂ 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*₄*)m*), JCGC801 (labelled Δ*c*₄) and JCGC820 (labelled *c*₄Δ*c*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*₅*)m*), JCGC860 (labelled Δ*c*₅) and JCGC870 (labelled *c*₅Δ*c*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) The presence of covalently bound heme in strains JCGC10 (labelled $c_4I$), JCGC801 (labelled $\Delta c_4$) and JCGC820 (labelled $c_4I \Delta c_4$) 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 $\Delta c_5$) and JCGC870 (labelled $c_5I \Delta c_5$) 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 $c_4$I), JCGC810 (labelled $\Delta c_4$) and JCGC820 (labelled $c_4I\Delta c_4$). 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 $c_5$I), JCGC860 (labelled $\Delta c_5$) and JCGC870 (labelled $c_5I\Delta c_5$). 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)

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<td>c₄lc₄ + IPTG</td>
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B

Rate of oxygen reduction (nmol O₂ reduced min⁻¹. mg dry cell mass⁻¹)

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P-values:

A: P = 8.13 x 10⁻⁵, P = 0.227
B: P = 5.80 x 10⁻⁶, P = 0.870
$c_2$ 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 ($\Delta$c₅ $\Delta$c₄ c₅I) 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.
The cytochrome \( \text{cbb}_3 \) 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 \( \text{cbb}_3 \) oxidases from various bacteria, and the structure of the cytochrome \( \text{cbb}_3 \) oxidase from \textit{Pseudomonas stutzeri} was recently published (Preisig \textit{et al.}, 1993; Garcia-Horsman \textit{et al.}, 1994a; Gray \textit{et al.}, 1994; de Gier \textit{et al.}, 1996; Preisig \textit{et al.}, 1996; Visser \textit{et al.}, 1997; Arslan \textit{et al.}, 2000; Pitcher & Watmough, 2004; Buschmann \textit{et al.}, 2010). Although soluble monoheme cytochromes transfer electrons between the cytochrome \( \text{bc}_1 \) complex and cytochrome \( \text{ca}_3 \) oxidase, little is known about how electrons are transferred from the cytochrome \( \text{bc}_1 \) complex to the cytochrome \( \text{cbb}_3 \) oxidase family.

### 3.7.1 Essential roles of either cytochrome \( c_4 \) or cytochrome \( c_5 \) in electron transfer between the gonococcal cytochrome \( \text{bc}_1 \) complex and cytochrome oxidase

Downs and Jones were among the first groups to propose that in \textit{Azotobacter vinelandii}, the diheme cytochrome \( c_4 \) and monoheme cytochrome \( c_5 \) might provide alternative but parallel pathways for electron transfer from the cytochrome \( \text{bc}_1 \) complex to the terminal oxidase (Downs & Jones, 1975). Much later, it was realized that there are multiple cytochrome oxidases in \textit{A. vinelandii} and that one of them is a homologue of the \textit{B. japonicum} high-affinity oxidase FixNOPQ (Kelly \textit{et al.}, 1990; Kolonay \textit{et al.}, 1994; Thöny-Meyer \textit{et al.}, 1994; D’mello \textit{et al.}, 1997). \textit{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
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 bc₁ 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.
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 $c_6$ 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 c₄ or c₅ 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 bc₁ 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 \( \text{cycA} \) and \( \text{cycB} \) 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 \( \text{cycA} \) and \( \text{cycB} \) mutants than in the parent strain due to their lower rates of electron transfer from the cytochrome \( \text{bc}_1 \) 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 c₂, 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 c₂ does not appear to be oxidised by the cytochrome cbb₃ 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 c₂ is similar to a domain of many copper-containing nitrite reductases. For example, cytochrome c₂ 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 c₂ 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 Neisseria 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.
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
Moraxella catarrhalis AniA amino acid sequence is labelled as Mc AniA, and the Neisseria gonorrhoeae AniA-cytochrome c₂ hybrid amino acid sequence is labelled as Ng AniA+c2. The AniA-cytochrome c₂ hybrid contains the full amino acid sequence of the AniA protein followed by residues 30-132 of cytochrome c₂ (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 c₂ 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 c₂ hybrid of *Neisseria gonorrhoeae*. The AniA-cytochrome c₂ hybrid contains the full amino acid sequence of the AniA protein followed by residues 30-132 of cytochrome c₂ (the latter indicated by underlined residues). Asterisks (*) indicate identical amino acids; colons (:) indicate conserved substitutions; and full stops (.) indicate semi-conserved substitutions.
Trimeric forms of AniA proteins from *Pseudoalteromonas haloplanktis* TAC125 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 ($\Delta c_2$), 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 (Δc₂)

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 (Δc₂) and JCGC140 (c₂I)

Strain JCGC140 contains the additional copy of the cccA gene under the control of the lac promoter at the lctP-aspC locus. Cytochrome c₂ 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 (Δc₂) and JCGC140 (c₂I)

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 c₂ band, although faint, is a doublet.
replaced with a kanamycin resistance cassette) and JCGC142 (cytochrome c\textsubscript{2} deletion mutant complemented with the ectopically expressed copy of cytochrome c\textsubscript{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\textsubscript{2}. The growth rate of strain JCGC142 is slightly improved on addition of IPTG, suggesting that the IPTG inducible copy of cytochrome c\textsubscript{2} is able to complement the loss of cytochrome c\textsubscript{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\textsubscript{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\textsubscript{2} (as shown in Figure 4.12) makes this result unexpected, as it seems that the cytochrome c\textsubscript{2} synthesised is unable to fully reduce the nitrite reductase, AniA. This phenomenon was not observed for the IPTG-inducible copies of cytochromes c\textsubscript{4} and c\textsubscript{5}, which were fully able to complement the c\textsubscript{4} and c\textsubscript{5} deletion mutants, respectively, as shown in Chapter 3. This
Figure 4.11 – Oxygen-limited, nitrite-supplemented growth of strains F62, JCGC851 (Δc₂) and JCGC142 (c₂I Δc₂)

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 (Δc₂) and JCGC142 (c₂I Δc₂)

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 (Δc₂) and JCGC142 (c₂I Δc₂)

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 c₂ 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 (Δc₂), JCGC807 (c₂ 3xFLAG) and JCGC805 (c₄ 3xFLAG).

A) Oxygen-limited nitrite-supplemented growth. JCGC807 encodes a 3xFLAG-tagged copy of cytochrome c₂, and JCGC805 encodes a 3xFLAG-tagged copy of cytochrome c₄, 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 c₄ 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 c₂ protein in strains F62, JGC851 (Δc₂) and JCGC807 (c₂ 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 c₂ protein in strain JCGC807 (encoding a 3xFLAG-tagged copy of cytochrome c₂ 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 \textit{lctP-aspC} locus and is under the control of the \textit{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 \textit{cccA} with the potential stem loop unmuted or mutated, respectively. Strains F62, JCGC851 ($\Delta c_2$), JCGC152 ($c_2$I TTC $\Delta c_2$) and JCGC153 ($c_2$I AAG $\Delta c_2$) 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.

\textbf{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 (Δc₂), JCGC152 (c₂I TTC Δc₂) and JCGC153 (c₂I AAG Δc₂)

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**

![Graph showing OD at 650 nm over time](chart.png)

- Blue line labeled F62
- Red line labeled Δc₂
- Green line labeled c₂I TTC Δc₂
- Black line labeled c₂I AAG Δc₂
- Purple line labeled c₂I TTC Δc₂ + IPTG
- Dark purple line labeled c₂I AAG Δc₂ + IPTG

Arrows indicate 1 mM and 4 mM at 1 hour.

**B**

![Graph showing nitrite levels over time](chart.png)

- Blue line labeled F62
- Red line labeled Δc₂
- Green line labeled c₂I TTC Δc₂
- Black line labeled c₂I TTC Δc₂ + IPTG
- Purple line labeled c₂I AAG Δc₂
- Dark purple line labeled c₂I AAG Δc₂ + IPTG

**C**

![Image of Western blot](image.png)

- Labeled with F62, Δc₂, c₂I TTC Δc₂, c₂I AAG Δc₂
- +/- IPTG

1 mM 4 mM
Figure 4.18 – SDS-PAGE gel stained for covalently bound heme in cultures of strains F62, JCGC851 (Δc2), JCGC152 (c2I TTC Δc2) and JCGC153 (c2I AAG Δc2)

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 strain 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 c2 would have been degraded.

4.8.2 The expression of cytochrome c2 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 c2 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 c2 expression by nitrite. Furthermore, the fact that cytochrome c2 still accumulates in the absence of nitrite suggests that cytochrome c2 is expressed at a low constitutive level.

4.8.3 The overexpression of cytochrome c2 cannot complement a cytochrome c2 deletion mutant

Although the overexpression of cytochrome c2 was successful, the system was unable to
complement the absence of the original copy of cytochrome c₂ 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 c₄ and c₅ by inducing expression of the relevant cytochromes with IPTG. This suggests that an excess of cytochrome c₂ 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 c₂ is particularly high, an increase in the concentration of cytochrome c₂ might oxidise the electron transfer pathway. The most similar, characterised c-type cytochrome to gonococcal cytochrome c₂ is cytochrome c₅₅₂ 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 c₂ would be sufficiently oxidizing to affect the redox balance in the periplasm. An alternative possibility is that cytochrome c₂ 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 c₂ 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 c₂?
Cytochrome c₂ is very important for the reduction of nitrite by the gonococcus. Therefore it would be expected that the stoichiometry of cytochrome c₂ to the other c-type cytochromes, such as cytochrome c₄, should be of the same order. It is therefore surprising that the abundance of cytochrome c₂ is very low. One possibility is that cytochrome c₂ 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 cd₁-type nitrite reductase, which receives electrons from two routes, namely cytochrome c₅₅₀ 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 (\( \Delta c_4 \)) and JCGC850 (\( \Delta c_5 \)) 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 ($\Delta c_4$) and JCGC850 ($\Delta c_5$)

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.
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<th>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</th>
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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 Neisseria 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 (\( \text{kan}^R \) 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 c₄.
Figure 5.5 – Aerobic growth and rates of oxygen reduction by CcoP third heme group mutants

A) Aerobic growth of strains AHGC100 (F62 kan<sup>R</sup>), 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<sup>-1</sup> (mg of bacterial biomass)<sup>-1</sup> ± 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

OD_{650\text{ nm}}

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)

F62  CcoP C368A  CcoP C371A  CcoP C371A H372A  CcoP C368A C371A H372A

\( P = 1.71 \times 10^{-5} \)  \( P = 0.654 \)  \( P = 0.269 \)  \( P = 0.386 \)
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 c₅

To determine whether CcoP and cytochrome c₅ were perhaps the only two electron donors to AniA, attempts were made to transfer the c₅ 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 c₅
Figure 5.6 – Oxygen-limited, nitrite-supplemented growth of CcoP third heme group mutants

A) Oxygen-limited growth of strains AHGC100 (F62 \textit{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.

\[ P = 1.58 \times 10^{-9}, P = 4.48 \times 10^{-7}, P = 0.002, P = 3.96 \times 10^{-4} \]
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$_5$∆200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c$_5$∆200-279) 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$_5$∆200-279) and JCGC320 (CcoP C368A c$_5$∆200-279) 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 (c₅Δ200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c₅Δ200-279). 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 ($c_5\Delta200-279$) and JCGC320 (CcoP C368A $c_5\Delta200-279$), 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 $c_5\Delta200-279$), 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 \(c_5\Delta\)) 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 \(c_5\Delta\)). 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 \(c_5\) \((c_5\Delta200-279)\) overlap in these figures.
Figure 5.10 – Rates of nitrite reduction and accumulation of AniA protein in strains F62, JCGC310 (c₅Δ200-279), AHGC101 (CcoP C368A) and JCGC320 (CcoP C368A c₅Δ200-279)

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 (c₅Δ200-279)

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 Δc₄) was 27% that of the parent strain, which is lower than the rate of nitrite reduction by the cytochrome c₄ 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 c₄ 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 c₂ deletion mutation into a CcoP C368A Δc₄ double mutant

It has been shown in previous chapters that cytochrome c₂ is required for nitrite reduction to take place at its maximum rate. To determine whether cytochrome c₂ forms part of the same pathway of electron transfer to AniA as the CcoP or cytochrome c₄ pathways, the cytochrome c₂ deletion mutation was transferred into strain JCGC340 (CcoP C368A Δc₄) to produce strain JCGC351 (CcoP C368A Δc₄ Δc₂). Oxygen-limited, nitrite-supplemented growth of this strain was compared to that of strains F62, AHGC101 (CcoP C368A), JCGC800 (Δc₄) and JCGC340. The deletion of cytochrome c₂ from strain JCGC340 (CcoP C368A Δc₄) 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 (∆c₂), JGC800 (∆c₄), JGC340 (CcoP C₃₆₈A ∆c₄) and JGC351 (CcoP C₃₆₈A ∆c₄ ∆c₂)

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.
**A**

OD$_{650}$ nm vs. Time (hours)

- F62
- CcoP C368A
- Δc$_4$
- CcoP C368A Δc$_4$
- CcoP C368A Δc$_4$ Δc$_2$

**B**

OD$_{650}$ nm vs. Time (hours)

- F62
- CcoP C368A
- Δc$_4$
- CcoP C368A Δc$_4$
- CcoP C368A Δc$_4$ Δc$_2$

**C**

Western blot images:

- F62
- CcoP
- Δc$_4$
- CcoP Δc$_4$
- CcoP Δc$_4$ Δc$_2$

1 mM

4 mM
Figure 5.13 – Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC800 (∆c₄), AHGC101 (CcoP C368A) and JCGC340 (CcoP C368A ∆c₄)

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 $\Delta c_2 \Delta c_4$ 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 $c_5 \Delta 200-279$ double mutant

The double mutant defective in the third heme group of CcoP (CcoP C368) and the second domain of cytochrome $c_5$ ($c_5 \Delta 200-279$) 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 $c_5 \Delta 200-279$) 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, JGCG320 (CcoP C368A c5Δ200-279), JGCG350 (CcoP C368A c5Δ200-279 Δc2) and JGCG200 (ΔaniA) 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 JGCG320 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 gonococcus 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 (∆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 c5Δ200-279). The rate of nitrite reduction of strain JCGC355 (CcoP C368A c5Δ200-279 ΔcycP) 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 (ΔcycP) and JCGC355 (CcoP C368A c5Δ200-279 ΔcycP)

A) Growth of strains F62 and JCGC214. Solid lines indicate the $OD_{650 \text{ nm}}$ 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**

Graph showing OD at 650 nm over time with data points for different concentrations of nitrite (1 mM and 4 mM). The graph includes lines for F62 and ΔcycP strains.

**B**

Bar graph comparing the rate of nitrite reduction (nmol NO₂⁻ reduced min⁻¹. mg dry cell mass⁻¹) for F62, ΔcycP, and ccoP C368A c₅∆200-279 ΔcycP strains. The graph shows the rate values with error bars. The statistical significance is indicated with $P = 2.39 \times 10^{-9}$ for F62 vs. ΔcycP and $P = 1.583 \times 10^{-6}$ for ccoP C368A c₅∆200-279 ΔcycP.

**C**

Image of protein expression levels for F62, ΔcycP, and CcoP c₅Δ strains, with Western blot analysis.
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 (Δ*c*2 Δ*laz*), JCGC335 (ccoPC368A Δ*laz*), JCGC317 (c5Δ200-279 Δ*laz*) and JCGC352 (ccoPC368A c5Δ200-279 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of nitrite reduction</th>
<th>No. replicates</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent (F62)</td>
<td>222 ± 8</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Δlaz</td>
<td>188 ± 7</td>
<td>7</td>
<td>0.004 (F62)</td>
</tr>
<tr>
<td>Δc₂</td>
<td>115 ± 7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Δc₂ Δlaz</td>
<td>121 ± 8</td>
<td>6</td>
<td>0.557 (Δc₂)</td>
</tr>
<tr>
<td>ccoP C368A</td>
<td>115 ± 8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>ccoP C368A Δlaz</td>
<td>114 ± 8</td>
<td>6</td>
<td>0.9351 (ccoP C368A)</td>
</tr>
<tr>
<td>c₅ Δ200-279</td>
<td>87 ± 9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>c₅ Δ200-279 Δlaz</td>
<td>64 ± 10</td>
<td>6</td>
<td>0.1214 (c₅ Δ200-279)</td>
</tr>
<tr>
<td>ccoP C368A c₅ Δ200-279</td>
<td>26 ± 1.3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>ccoP C368A c₅ Δ200-279 Δlaz</td>
<td>27 ± 1</td>
<td>6</td>
<td>0.6744 (ccoP C368A c₅ Δ200-279)</td>
</tr>
</tbody>
</table>

Rates of nitrite reduction by washed bacterial suspensions of strains F62, JCGC890 (Δlaz), JCGC851 (Δc₂), JCGC892 (Δc₂ Δlaz), AHGC101 (ccoP C368A), JCGC335 (ccoP C368A Δlaz), JCGC310 (c₅Δ200-279), JCGC317 (c₅Δ200-279 Δlaz), JCGC320 (ccoP C368A c₅Δ200-279) and JCGC352 (ccoP C368A c₅Δ200-279 Δlaz). 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 Δ$c_2$), JCGC800 (labelled Δ$c_4$), JCGC853 (labelled Δ$c_4$ Δ$c_2$), JCGC850 (labelled Δ$c_5$), JCGC852 (labelled Δ$c_5$ Δ$c_2$), JCGC310 (labelled Δ$c_5$Δ), JCGC315 (labelled Δ$c_5$Δ Δ$c_2$), AHGC101 (CcoP), JCGC330 (CcoP Δ$c_2$), JCGC890 (Δ$laz$), JCGC892 (Δ$laz$ Δ$c_2$), JCGC214 (Δ$cycP$) and JCGC216 (Δ$cycP$ Δ$c_2$) during oxygen-limited growth detected by Western blotting. Samples were taken after 5 hours of growth.
5.13  Role of cytochrome c₂ in electron transfer to AniA

When the cytochrome c₂ coding region was deleted from strains JCGC340 (CcoP C368A Δc₄) and JCGC320 (CcoP C368A Δc₅) to produce strains JCGC351 and JCGC350, respectively, the deletion of cytochrome c₂ resulted in a decreased nitrite reduction rate in both strains. This suggests that the cytochrome c₂ electron transfer pathway to AniA is independent of the CcoP, cytochrome c₄ and cytochrome c₅ pathways. To further dissect the electron transfer pathways to AniA, cytochrome c₂ deletion mutations were added to strains JCGC800 (Δc₄), JCGC850 (Δc₅), JCGC310 (c₅ Δ200-279) and AHGC101 (CcoP C368A) to produce strains JCGC853 (Δc₄ Δc₂), JCGC852 (Δc₅ Δc₂), JCGC315 (c₅ Δ200-279 Δc₂) and JCGC330 (CcoP C368A Δc₂), respectively. The deletion of cytochrome c₂ from strains JCGC850 (Δc₅) and JCGC310 (c₅Δ200-279) resulted in a 66% and 60% decrease in nitrite reduction rate, respectively (Table 5.2). This suggests that the cytochrome c₂ pathway of electron transfer to AniA is independent of the cytochrome c₅ pathway. The deletion of cytochrome c₂ from strain JCGC800 (lacking cytochrome c₄) resulted in a slightly increased rate of nitrite reduction, but the difference was not statistically significant (Table 5.2). This suggests that cytochromes c₂ and cytochrome c₄ 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 c₂ 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 c₂. 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of nitrite reduction</th>
<th>No. replicates</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent (F62)</td>
<td>222 ± 8</td>
<td>29</td>
<td>1.37 x 10^{-12} (F62)</td>
</tr>
<tr>
<td>$\Delta c_2$</td>
<td>115 ± 7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>$\Delta c_4$</td>
<td>81 ± 6</td>
<td>7</td>
<td>1.66 x 10^{-13} (F62)</td>
</tr>
<tr>
<td>$\Delta c_4 \Delta c_2$</td>
<td>93 ± 5</td>
<td>8</td>
<td>0.17 ($\Delta c_4$)</td>
</tr>
<tr>
<td>$\Delta c_5$</td>
<td>130 ± 7</td>
<td>8</td>
<td>1.37 x 10^{-9} (F62)</td>
</tr>
<tr>
<td>$\Delta c_5 \Delta c_2$</td>
<td>86 ± 9</td>
<td>11</td>
<td>9.136 x 10^{-5} ($\Delta c_5$)</td>
</tr>
<tr>
<td>$\Delta c_5 \Delta 200$-279</td>
<td>87 ± 9</td>
<td>6</td>
<td>1.26 x 10^{-8} (F62), 0.003 ($\Delta c_5$)</td>
</tr>
<tr>
<td>$\Delta c_5 \Delta 200$-279 $\Delta c_2$</td>
<td>52 ± 4</td>
<td>6</td>
<td>0.01 ($\Delta c_5 \Delta 200$-279)</td>
</tr>
<tr>
<td>$c_5 \Delta 200$-279 $\Delta c_2$</td>
<td>115 ± 8</td>
<td>8</td>
<td>1.58 x 10^{-9} (F62)</td>
</tr>
<tr>
<td>$c_5 \Delta 200$-279 $\Delta c_2$</td>
<td>115 ± 15</td>
<td>5</td>
<td>0.972 ($c_5 \Delta 200$-279)</td>
</tr>
<tr>
<td>$\Delta laz \Delta c_2$</td>
<td>122 ± 8</td>
<td>6</td>
<td>0.012 ($\Delta laz$)</td>
</tr>
<tr>
<td>$\Delta cycP$</td>
<td>121 ± 7</td>
<td>7</td>
<td>2.39 x 10^{-9} (F62)</td>
</tr>
<tr>
<td>$\Delta cycP \Delta c_2$</td>
<td>115 ± 6</td>
<td>6</td>
<td>0.52 ($\Delta cycP$)</td>
</tr>
</tbody>
</table>

Rates of nitrite reduction by washed bacterial suspensions of strains F62, JGC851 ($\Delta c_2$), JGC850 ($\Delta c_4$), JGC853 ($\Delta c_4 \Delta c_2$), JGC850 ($\Delta c_5$), JGC852 ($\Delta c_5 \Delta c_2$), JGC851 ($\Delta c_5 \Delta 200$-279), JGC315 ($c_5 \Delta 200$-279 $\Delta c_2$), AHGC101 ($c_5 \Delta 200$-279 $\Delta c_2$), JGC330 ($c_5 \Delta 200$-279 $\Delta c_2$), JGC890 ($\Delta laz$), JGC892 ($\Delta laz \Delta c_2$), JGC214 ($\Delta cycP$) and JGC216 ($\Delta cycP \Delta c_2$). 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 $b_{c_1}$ 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 $c_2\Delta 200-279$) 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 c₄ is known to transfer electrons to the cytochrome cbb₃ complex, and, based on the solved structure of the cytochrome cbb₃-type oxidase from *Pseudomonas stutzeri*, both cytochromes c₄ and c₅ 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 c₄ and the first heme group of cytochrome c₅ contribute indirectly to electron flux to AniA via the third heme group of CcoP. It is likely that cytochrome c₄ contributes relatively more electrons to the CcoP subunit, as a proportion of the electrons that pass through cytochrome c₅ are diverted to the second heme group of cytochrome c₅, which has been shown to be an electron donor to AniA. One could therefore speculate that the decreased nitrite reduction rate in the cytochrome c₄ 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 c₄ means that only the first heme group of cytochrome c₅ 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 c₅ 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 c₅.

5.14.5 Poor growth of the triple mutant lacking cytochromes c₄, c₂ 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 $c_5\Delta 200\text{-}279$) 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 $\Delta cycP$ 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 \(c_3\Delta200-279\)) 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*<sub>3</sub>-type, a cytochrome *bc<sub>1</sub>* complex, and cytochromes *c<sub>2</sub>*, *c<sub>4</sub>* and *c<sub>5</sub>* all of which were obvious candidates for electron transfer components to oxygen. It was shown in Chapter 3 that, as expected, cytochromes *c<sub>4</sub>* and *c<sub>5</sub>* form a bifurcated pathway from the cytochrome *bc<sub>1</sub>* complex to the *cbb*<sub>3</sub>-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<sub>4</sub>* *c<sub>5</sub>* double mutant could be constructed in this bacterium only when an ectopic copy of the cytochrome *c<sub>5</sub>* gene was present, the expression of which could be induced by IPTG.

The redox potentials of the gonococcal cytochromes *c<sub>4</sub>* and *c<sub>5</sub>* 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<sup>-1</sup>K<sup>-1</sup>)
- \( T = \) temperature (K)
- \( n = \) number of electrons
- \( F = \) Faraday constant (96,485 Coulombs mol<sup>-1</sup>)
- \( Q = \) 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>
<thead>
<tr>
<th>Organism</th>
<th>Redox protein/component</th>
<th>Redox couple</th>
<th>$E'_m$ (mV)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dehydrogenases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> dehydrogenase</td>
<td>NADH</td>
<td>NAD$^+$/NADH</td>
<td>-320</td>
<td>(Unden &amp; Bongaerts, 1997)</td>
</tr>
<tr>
<td><em>E. coli</em> succinate dehydrogenase</td>
<td>Succinate</td>
<td>fumarate/ succinate</td>
<td>+30</td>
<td>(Unden &amp; Bongaerts, 1997)</td>
</tr>
<tr>
<td><strong>Quinol/cytochrome bc$_1$ complex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Ubiquinol</td>
<td>UQ/UQH$_2$</td>
<td>+90</td>
<td></td>
<td>(Snyder &amp; Trumpower, 1998)</td>
</tr>
<tr>
<td>$b_l$</td>
<td>$b_l$ (+3)/$b_l$ (+2)</td>
<td>-30</td>
<td></td>
<td>(Covian et al., 2004)</td>
</tr>
<tr>
<td>$b_H$</td>
<td>$b_H$ (+3)/$b_H$ (+2)</td>
<td>+90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rieske protein</td>
<td>2Fe$<em>{2S}^+$/2Fe$</em>{2S}$</td>
<td>+280*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_1$</td>
<td>Fe (+3)/ Fe (+2)</td>
<td>+270</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. sphaeroides</em> Ubiquinol</td>
<td>UQ/UQH$_2$</td>
<td>+90</td>
<td></td>
<td>(Bowyer &amp; Crofts, 1981; Crofts et al., 2003)</td>
</tr>
<tr>
<td>$b_l$</td>
<td>$b_l$ (+3)/$b_l$ (+2)</td>
<td>-90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_H$</td>
<td>$b_H$ (+3)/$b_H$ (+2)</td>
<td>+50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rieske protein</td>
<td>2Fe$<em>{2S}^+$/2Fe$</em>{2S}$</td>
<td>+310*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_1$</td>
<td>Fe (+3)/ Fe (+2)</td>
<td>+270</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cbb$_3$-type oxidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. sphaeroides</em> $c$ (CcoO, 2xCcoP)</td>
<td>Fe (+3)/ Fe (+2)</td>
<td>+234, +320, +351</td>
<td></td>
<td>(Rauhamaki et al., 2009)</td>
</tr>
<tr>
<td>$b$ (CcoN)</td>
<td>$b$ (+3)/$b$ (+2)</td>
<td>+418</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_3$ (CcoN)</td>
<td>$b_3$ (+3)/$b_3$ (+2)</td>
<td>-59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu$_b$ (CcoN)</td>
<td>Cu (+2)/Cu (+1)</td>
<td>+330</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. stutzeri</em> $c$ (CcoP)</td>
<td>Fe (+3)/ Fe (+2)</td>
<td>+185, +205, +245, +215</td>
<td></td>
<td>(Pitcher &amp; Watmough, 2004)</td>
</tr>
<tr>
<td>$c$ (CcoP)</td>
<td></td>
<td>+245, +205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c$ (CcoO)</td>
<td></td>
<td>+245, +215</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b$ (CcoN)</td>
<td>$b$ (+3)/$b$ (+2)</td>
<td>+310, +265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b_3$ (CcoN)</td>
<td>$b_3$ (+3)/$b_3$ (+2)</td>
<td>+225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*-$$O$_2^+ + 2H^+$ / H$_2$O$^-$</td>
<td></td>
<td>+820</td>
<td></td>
<td>(Pitcher &amp; Watmough, 2004)</td>
</tr>
<tr>
<td><strong>Copper-containing nitrite reductases (CuNiR)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. sphaeroides</em> Type 1 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+247</td>
<td></td>
<td>(Olesen et al., 1998)</td>
</tr>
<tr>
<td>Type 2 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>&lt;=200**</td>
<td></td>
<td>(Pinho et al., 2004)</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> Type 1 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+298</td>
<td></td>
<td>(Leferink et al., 2011)</td>
</tr>
<tr>
<td>Type 2 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+172**</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. xylosoxidans</em> Type 1 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+255</td>
<td></td>
<td>(Berk &amp; Watmough, 1995)</td>
</tr>
<tr>
<td>Type 2 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+244**</td>
<td></td>
<td>(Ainscough et al., 1987)</td>
</tr>
<tr>
<td>*-$$NO$_2^- /NO$</td>
<td></td>
<td>+375</td>
<td></td>
<td>(Taniguchi et al., 1980)</td>
</tr>
<tr>
<td><strong>Azurins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. denitrificans</em> Type 1 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+276</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> Type 1 Cu</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+308</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Component</td>
<td>Redox State</td>
<td>Potential (mV)</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------------------</td>
<td>-------------</td>
<td>----------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td><em>P. denitrificans</em></td>
<td>Type 1 Cu (Azurin)</td>
<td>Cu (+2)/Cu(+1)</td>
<td>+ 230</td>
<td>(Martinkus <em>et al.</em>, 1980)</td>
</tr>
<tr>
<td><strong>c-type cytochromes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>cytochrome <em>c</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 240</td>
<td>(Chang <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 340</td>
<td>(Tissieres, 1956)</td>
</tr>
<tr>
<td><em>A. vinelandii</em></td>
<td>cytochrome <em>c</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 300</td>
<td>(Kadziola &amp; Larsen, 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 241</td>
<td>(Carter <em>et al.</em>, 1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 328</td>
<td>(Korszun &amp; Salemme, 1977)</td>
</tr>
<tr>
<td><em>C. thiosulfatophilum</em></td>
<td>cytochrome <em>c</em>555*</td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 145</td>
<td>(Ogawa <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>S. violacea</em></td>
<td>cytochrome <em>c</em>552*</td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 202</td>
<td>(Fee <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td><strong>Cytochromes c’</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes</em> sp.*</td>
<td>cytochrome <em>c’</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 130</td>
<td>(Meyer <em>et al.</em>, 1986)</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>cytochrome <em>c’</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 30</td>
<td>(Meyer <em>et al.</em>, 1986)</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>cytochrome <em>c’</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 180</td>
<td>(Meyer <em>et al.</em>, 1983)</td>
</tr>
<tr>
<td><strong>Cytochrome c peroxidases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>cytochrome <em>c</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 320</td>
<td>(Ellfolk <em>et al.</em>, 1983)</td>
</tr>
<tr>
<td></td>
<td>cytochrome <em>c</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>- 330</td>
<td></td>
</tr>
<tr>
<td><em>N. europaea</em></td>
<td>cytochrome <em>c</em></td>
<td>Fe (+3)/Fe (+2)</td>
<td>+ 450</td>
<td>(Arciero &amp; Hooper, 1994)</td>
</tr>
<tr>
<td></td>
<td>cytochrome c</td>
<td>Fe (+3)/Fe (+2)</td>
<td>- 258</td>
<td>(Bertagna <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>H₂O₂ / 2H₂O</td>
<td>+ 1770</td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Ascorbate</td>
<td>dehydroascorbic acid/ascorbic acid</td>
<td>+ 58</td>
<td>(Weis, 1975)</td>
</tr>
<tr>
<td></td>
<td>TMPD</td>
<td>TMPD⁺/TMPD</td>
<td>+ 260</td>
<td>(Jurtshuk <em>et al.</em>, 1975)</td>
</tr>
</tbody>
</table>

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 $c_5$ 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 $c_5$ 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of nitrite reduction</th>
<th>No. replicates</th>
<th>% parental rate</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent (F62)</td>
<td>222 ± 8</td>
<td>29</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>ΔccoP</td>
<td>115 ± 8</td>
<td>8</td>
<td>52%</td>
<td>1.58 x 10^-9 (F62)</td>
</tr>
<tr>
<td>Δc₃</td>
<td>130 ± 7</td>
<td>8</td>
<td>59%</td>
<td>1.37 x 10^-9 (F62)</td>
</tr>
<tr>
<td>c₅Δ200-279</td>
<td>87 ± 9</td>
<td>6</td>
<td>39%</td>
<td>1.26 x 10^-8 (F62), 0.003 (Δc₅)</td>
</tr>
<tr>
<td>c₅Δ200-279 ΔccoP</td>
<td>26 ± 1.3</td>
<td>16</td>
<td>12%</td>
<td>0.001 (c₅Δ200-279)</td>
</tr>
<tr>
<td>Δc₂</td>
<td>115 ± 7</td>
<td>19</td>
<td>52%</td>
<td>1.37 x 10^-12 (F62)</td>
</tr>
<tr>
<td>Δc₂ Δc₅</td>
<td>86 ± 9</td>
<td>11</td>
<td>39%</td>
<td>9.136 x 10^-4 (Δc₂)</td>
</tr>
<tr>
<td>Δc₂ c₅Δ200-279</td>
<td>52 ± 4</td>
<td>6</td>
<td>23%</td>
<td>0.01 (c₅Δ200-279)</td>
</tr>
<tr>
<td>Δc₂ ΔccoP</td>
<td>115 ± 15</td>
<td>5</td>
<td>52%</td>
<td>0.972 (ΔccoP)</td>
</tr>
<tr>
<td>Δc₂ ΔccoP c₅Δ200-279</td>
<td>17 ± 3</td>
<td>6</td>
<td>8%</td>
<td>0.0192 (ΔccoP)</td>
</tr>
<tr>
<td>Δc₄</td>
<td>81 ± 6</td>
<td>7</td>
<td>36%</td>
<td>1.66 x 10^-10 (F62)</td>
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<tr>
<td>Δc₄ Δc₂</td>
<td>93 ± 5</td>
<td>8</td>
<td>42%</td>
<td>0.17 (Δc₄)</td>
</tr>
<tr>
<td>Δc₄ ΔccoP</td>
<td>79 ± 7.7</td>
<td>10</td>
<td>36%</td>
<td>0.8069 (Δc₄), 0.004 (ΔccoP)</td>
</tr>
<tr>
<td>Δc₄ ΔccoP Δc₂</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ΔcycP</td>
<td>121 ± 7</td>
<td>7</td>
<td>55%</td>
<td>2.39 x 10^-9 (F62)</td>
</tr>
<tr>
<td>ΔcycP Δc₂</td>
<td>115 ± 6</td>
<td>6</td>
<td>52%</td>
<td>0.52 (ΔcycP)</td>
</tr>
<tr>
<td>ΔcycP ΔccoP c₅Δ200-279</td>
<td>20 ± 3</td>
<td>6</td>
<td>9%</td>
<td>0.078 (ΔccoP c₅)</td>
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<tr>
<td>Δlaz</td>
<td>188 ± 7</td>
<td>7</td>
<td>84%</td>
<td>0.004 (F62)</td>
</tr>
<tr>
<td>Δlaz c₅Δ200-279</td>
<td>64 ± 10</td>
<td>6</td>
<td>29%</td>
<td>0.1214 (c₅ Δ200-279)</td>
</tr>
<tr>
<td>Δlaz Δc₅</td>
<td>122 ± 8</td>
<td>6</td>
<td>55%</td>
<td>0.012 (Δlaz)</td>
</tr>
<tr>
<td>Δlaz ΔccoP</td>
<td>114 ± 8</td>
<td>6</td>
<td>51%</td>
<td>0.9351 (ΔccoP)</td>
</tr>
<tr>
<td>Δlaz ΔccoP c₅Δ200-279</td>
<td>27 ± 1</td>
<td>6</td>
<td>12%</td>
<td>0.6744 (ΔccoP c₅)</td>
</tr>
<tr>
<td>ΔaniA</td>
<td>1 ± 0.4</td>
<td>8</td>
<td>0.004%</td>
<td>0.002 (ΔccoP c₅)</td>
</tr>
</tbody>
</table>

Units for rates of nitrite reduction are nmol NO₂⁻ 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. ΔccoP = 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 c5) were known, it may aid in the prediction of the electron donor to the third heme group of CcoP. The fact that cytochromes c4 and c5 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 c5 is a major electron donor to AniA

The rate of nitrite reduction by a cytochrome c5 deletion mutant was 59% that of the parent strain (Table 6.2), implicating cytochrome c5 as an electron donor to AniA. The third heme-binding domain of CcoP is similar to the second heme-binding domain of cytochrome c5, which indicated a role for the second heme group of cytochrome c5 as an electron donor to AniA. Results from Chapter 5 suggest that the two domains of cytochrome c5 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 c5 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 c₄ mutant, which suggested that cytochrome c₄ might be the only electron donor to CcoP. However, if this were true the rate of nitrite reduction of strains JCGC800 (∆c₄) and AHGC101 (CcoP C368A) would be identical, which they are not (Table 6.2). The deletion of cytochrome c₄ leaves cytochrome c₅ as the only electron donor to the second heme group of CcoP, so cytochrome c₅ would be the only electron donor to oxygen via CcoO and nitrite via the third heme group of CcoP. The lack of cytochrome c₄ therefore restricts the electron flow to the terminal oxidase and to AniA due to the bifunctionalism of cytochrome c₅. 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 c₄ and c₅ could be donated to AniA at different stages in the electron transfer chain, and therefore means that, in the absence of oxygen, cytochrome c₄ 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 cbb₃-type terminal oxidase (which has a low Kₘ 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 ($\text{CcoP C368A c}_5\Delta200$-$279$) 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 ($\text{CcoP C368A c}_2\Delta200$-$279$), 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 $\text{laz}$ or $\text{cycA}$ (encoding cytochrome $c_4$) genes from strain JCGC350 ($\text{CcoP C368A c}_5\Delta200$-$279$ $\Delta c_2$) 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 ($c_5\Delta200$-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 ($\Delta c_4$), 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 c₅ 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 c₅ 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 c₅ 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 $cd_2$-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 Scol 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 Scol 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 Scol homologue, which could aid in the biogenesis of the copper centres of the nitrite reductase. Although Scol 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 Scol 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 Scol, 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<sub>2</sub> might increase the competition between cytochrome c<sub>2</sub> and cytochrome c<sub>5</sub> for binding to the terminal oxidase, resulting in a greater flux of electrons to the second heme group of cytochrome c<sub>5</sub>. In theory, this should increase the flow of electrons to AniA and therefore increase the rate of nitrite reduction of the complemented cytochrome c<sub>2</sub> mutant. However, the increase in competition between cytochromes c<sub>5</sub> and c<sub>2</sub> could result in a lower electron flux to the third heme group of CcoP as fewer electrons would available to CcoP from cytochrome c<sub>5</sub>. 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<sub>2</sub>, the resulting increase in electron flux from the second heme group of cytochrome c<sub>5</sub> 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<sub>2</sub> deletion mutant would not be restored to that of the parent strain by complementation with the NICS system, due to the excess cytochrome c<sub>2</sub> 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 c₅, AniA, Laz (lipid-associated azurin), Scol 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 Scol 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 c₂ might have previously existed as a domain of the AniA protein. Interestingly, none of the AniA-cytochrome c₂ 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 c₂ 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.


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