Structural biology of the fatty acid biosynthesis (FASI) pathway of pathogenic Neisseria; a promising target for combating emerging drug resistance

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Cover image

Model of FabI from *Neisseria meningitidis* in complex with the inhibitor epigallocatechin gallate (yellow).
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Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

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Name

Jeffrey Nanson

Signature

Date
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-enzyme A</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory concentration; concentration required for 50% inhibition in vitro</td>
</tr>
<tr>
<td>LIC</td>
<td>Ligation independent cloning</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistant</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC at which 50% of bacterial isolates in test population are inhibited</td>
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<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC at which 90% of bacterial isolates in test population are inhibited</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>P-pant</td>
<td>4′-phosphopantetheine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phosphine hydrochloride</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
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Abstract

*Neisseria* is a large genus of Proteobacteria that colonize the mucosal surfaces of both humans and animals. Most *Neisseria* spp. are considered to be non-pathogenic commensal bacteria, with only *Neisseria gonorrhoeae* and *Neisseria meningitidis* being regarded as human pathogens. Despite sharing a large number of genes and virulence factors, these two closely related obligate human pathogens cause distinctive diseases.

Gonorrhoea is a sexually transmissible infection caused by the bacterium *Neisseria gonorrhoea*. This infection is of major health concern due to high rates of disease, and rapidly diminishing treatment options due to drug resistance. Ceftriaxone is the current first line treatment in most countries, with no identified ideal alternative. The emergence of multiple drug resistant (MDR) strains with decreased sensitivity to ceftriaxone has been reported in many countries, including Australia. Reports of MDR strains of *N. gonorrhoeae* resistant to ceftriaxone, termed extensively drug resistant (XDR), have caused considerable alarm. The World Health Organisation has issued a Global Action Plan to minimise the spread and impact of antimicrobial resistance in *N. gonorrhoeae*, calling for the development of new treatments as an urgent priority. In 2013, the USA Centres for Disease Control and Prevention classified *N. gonorrhoeae* drug resistance in the top three of antibiotic resistance threats. There are no vaccines available for prevention of *N. gonorrhoeae* infections, thus the current treatment is antibiotic therapy. With no ideal alternative treatment or vaccine available, it is widely feared that gonorrhoea will become untreatable.

Antimicrobial resistance in *N. meningitidis*, the causative agent of debilitating and potentially fatal meningococcal meningitis, also appears to be increasing. Whilst not as prevalent as in *N. gonorrhoeae*, resistance to penicillins, rifampicin, tetracyclines, and
sulphonamides is now widely reported in *N. meningitidis*, with at least one reported case of *N. meningitidis* displaying reduced susceptibility to the extended-spectrum cephalosporins ceftriaxone and cefotaxime. The drug resistance displayed by these pathogenic bacteria demonstrates an urgent need for development of new antimicrobial agents.

The fatty acid synthesis type II (FASII) enzymes of *N. gonorrhoeae* and *N. meningitidis*, responsible for lipid biogenesis, represent an attractive target for the development of new antimicrobials. Triclosan, a widely used antibacterial, inhibits this pathway, and demonstrates the validity of enzymes of the FASII pathway as drug targets. To provide a strong platform for the development of urgently needed antimicrobials, the three-dimensional structures of the FASII enzymes ACP synthase, FabD, FabF, FabG, FabH, and FabI from *N. meningitidis* (98-100% identity to those of *N. gonorrhoeae*) were solved by X-ray crystallography. Significantly, the FASII reductases FabG and FabI are inhibited by the plant polyphenol epigallocatechin gallate (EGCG), thus the structures of FabI in complex with EGCG, NADH, and NAD:triclosan, and FabG in complex with NADPH, were also determined, providing detailed knowledge of the active and co-factor binding sites within both enzymes. The binding sites for EGCG and NADH in FabI appear highly similar to those in FabG, possibly providing a basis for the design of antimicrobials that simultaneously target both enzymes, and are suitable for the treatment of drug resistant *N. gonorrhoeae* and *N. meningitidis*.
CHAPTER 1 - INTRODUCTION

First identified by Albert Neisser in 1879, Neisseria is a large genus of Proteobacteria that colonize the mucosal surfaces of both humans and animals. Of the 11 Neisseria spp. known to colonise humans, most are considered non-pathogenic commensal bacteria, with only Neisseria gonorrhoeae and Neisseria meningitidis being regarded as pathogenic (Kampmeier 1978, Zeigler 2003).

N. gonorrhoeae and N. meningitidis are two closely related obligate human pathogens that despite sharing a large number of genes and virulence factors are capable of causing distinct diseases; N. meningitidis, known primarily as a major cause of bacterial meningitis and septicaemia, and N. gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhoea (Tinsley et al. 1996).

1.1 Neisseria meningitidis

N. meningitidis is a common commensal bacterium, which colonizes the oronasopharynx of approximately 5-30% of healthy individuals (Caugant et al. 1994, Claus et al. 2005). A significantly higher carriage rate is observed in young adults in high population areas such as schools, universities, and army barracks, with carriage frequencies of up to 55% reported in one university in the United Kingdom (Ala'aldeen et al. 2011). Duration of the carriage state can last days to months depending on the colonizing strain, with one study reporting a carriage duration of at least 23 weeks in 26.7% of German teenagers (aged 15-18) (Glitza et al. 2008).

N. meningitidis is classified based on antigenic variations in the polysaccharide capsule that forms the outermost structure on the meningococcal surface. Of the 12 serogroups (A, B, C, E, H, I, K, L, Y, W, X and Z) identified, only six (A, B, C, W, X, Y) are
known to be associated with invasive disease (Harrison et al. 2013). In addition to serogroups, strains are further classified based on antigenic variable regions of the class 1 outer membrane protein (PorA), the iron-regulated outer membrane protein (FetA), and the allelic profile or multilocus sequence typing (MLST) of seven housekeeping genes, with sequence types assigned a clonal complex designation (serogroup: PorA type: FetA type: sequence type). In the case of ambiguous classification, additional characterisation also includes genotyping of the outer membrane protein PorB; PenA, the gene encoding the target of β-lactam type antibiotics, penicillin binding protein 2; factor H-binding protein (fHbp), a ubiquitously expressed virulence factor that binds to human factor H and prevents complement activation on the bacterial surface; Neisseria adhesin A (NadA), involved in host cell adhesion and invasion; and the Neisseria heparin-binding antigen (NHBA), known to confer resistance to serum-mediated killing (Thulin et al. 2006, Lucidarme et al. 2010, Serruto et al. 2010, McNeil et al. 2013).

The *N. meningitidis* polysaccharide capsule provides the basis of licensed polysaccharide-protein conjugate vaccines available for serogroups A, C, W, and Y. The polysaccharide capsule is composed of repeating units of (α2→8)-linked and (α2→9)-linked N-acetylmuraminic acid homopolymers in serogroups B and C respectively, D-galactose or D-glucose (α2→9)-linked N-acetylmuraminic acid in serogroups W and Y respectively, O-acetylated (α1→6)-linked N-acetyl-D-mannosamine-1-phosphate in serogroup A, and (α1→4)-linked N-acetylglucosamine-1-phosphate in the case of serogroup X (Jennings et al. 1977). Unlike the capsular polysaccharide serogroups A, C, W, and Y, the serogroup B capsular polysaccharide has proven to be a poor target for vaccine development because of a poor immunogenic response, presumably due to similarities between the (α2→8)-linked N-acetylmuraminic acid of the capsule and the polysialic acid containing glycopeptides
within human neural cells (Finne et al. 1983), with attempts to improve the immunogenic response likely hampered by fears of inappropriate immune response due to cross-reactivity.

In light of this, a number of highly conserved meningococcal surface antigens have been investigated in attempts to identify suitable epitopes to target serogroup B. This led to the development of the multicomponent meningococcal serogroup B (4CMenB) vaccine, which has recently been approved for use in the European Union, Australia, and Canada. Comprised of *N. meningitidis* outer membrane proteins PorA and PorB, virulence factors NadA, fHbp, and NHBA, and lipooligosaccaride (LOS), 4CMenB has shown strong immunological response and effectiveness in conferring protection against type B *N. meningitidis* strains, with some adverse effects such as mild to high fever reported in infants (Bjune 1991, O'Ryan et al. 2014).

Since their introduction, meningococcal vaccines have successfully caused significant reduction in both the incidence of invasive disease and carriage rate, with two significant limitations in that vaccines have reduced effectiveness in the very young, the most high risk age group in terms of invasive disease, as well as incomplete coverage of all Neisserial serogroups and strains. Vaccines are targeted towards preventing infections of certain serotypes. Currently there are no available vaccines that confer protection from *N. meningitidis* serogroup X (Micoli et al. 2013). Additionally, current licensed vaccines are predicted to induce specific bactericidal antibodies against as little as 66% of Neisserial strains within the target serogroup, depending on country and vaccine (Donnelly et al. 2010, O'Ryan et al. 2014). The limitations of these vaccines combined with the natural ability of *N. meningitidis* to change serogroup during an outbreak by horizontal transfer of genes encoding the polysaccharide capsule.
components arouses concern that their use may cause short term decrease in the incidence of invasive disease and confer immunity to specific serotypes and strains without lowering the long term incidence of meningococcal disease, thus increasing the incidence of serogroups and strains for which no vaccine is available (Vogel et al. 2000, Wang et al. 2010).

There is some evidence to suggest that long-term carriage can lead to invasive *N. meningitidis* infection, yet most infections are characterised by a rapid onset (1-14 days) and progression of symptoms. Despite the high incidence of *N. meningitidis* carriage in some populations, the incidence of non-asymptomatic *N. meningitidis* infections is relatively rare in Australia. The annual incidence of invasive *N. meningitidis* in Australia is approximately 1 in 100,000, and up to 14 in 100,000 in high risk groups such as infants and children under 5 (Lahra et al. 2013), is comparable to that observed in North America, Europe and New Zealand. The incidence of disease in Australia has continued to decrease in the past decade; however, the global incidence of invasive meningococcal infection varies due to localised outbreaks, epidemics, and pandemics. Currently, such epidemic and pandemic cases are restricted to the “meningitis belt” of sub-Saharan Africa, which extends across Ethiopia to Senegal (Harrison et al. 2009, Jafri et al. 2013).

1.1.1 Adherence, invasion and intracellular survival of *Neisseria meningitidis*

Transmission of *N. meningitidis* occurs through inhalation of respiratory droplets and secretions. Once inhaled, the bacterium adhere to the non-ciliated epithelial cells within the mucosal surface of the upper respiratory tract and begin to colonize the mucosal surface. *N. meningitidis* is able to attach to these epithelial cells via adhesion molecules, including the type IV pili (Tfp), the major outer membrane proteins Opa and Opc, the
LOS, factor H-binding protein (fHbp), PorA, PorB, and NadA, some of which extend beyond the polysaccharide capsule and bind to epithelial cell surface receptors such as CD46 and carcinoembryonic antigen-related cell-adhesion molecule 1 (CEACAM1).

Once established within the upper respiratory tract, *N. meningitidis* can cause localised infections such as pneumonia, sinusitis, or otitis media, or transverse the epithelial cells into the vasculature. Following initial bacterial adhesion to endothelial cells, Tfp induces the formation of cortical plaques and recruitment of several proteins to the site of bacterial adhesion, including the microfilamentous protein actin, leading to the formation of pseudopodia and internalisation of the meningococci (Merz et al. 1999). Following internalisation, *N. meningitidis* crosses the epithelial cell barrier by a transcellular route, entering into the vasculature and blood via an unknown mechanism believed to involve the microtubule network of the host epithelial cell. Ultimately, the bacteria disseminates throughout the body, leading to septicaemia or infection at other anatomical sites, resulting in conjunctivitis, arthritis, pericarditis or myocarditis (Sutherland et al. 2010).

Septicaemia is characterised by rapid proliferation of bacterium within the blood stream, as well as high concentrations of the meningococcal endotoxin LOS and outer membrane antigens that are shed in outer membrane vesicle blebs (Devoe et al. 1973, Pathan et al. 2003, Lee et al. 2007). The resulting intravascular inflammatory response causes progressive vascular damage, leading to severe disseminated coagulopathy, thrombotic lesions, circulatory collapse, and if unsuccessfully treated, multiple organ failure and death (Pathan et al. 2003).
Disseminated *N. meningitidis* can also enter into the meninges and the cerebrospinal fluid within the subarachnoid space (the space between the arachnoid and the pia mater), inducing inflammation, and causing the characteristic meningitis for which it is named. Penetration of the meninges and blood brain barrier occurs by a different mechanism than that of the upper respiratory tract, although both appear to be triggered by Tfp. *N. meningitidis* reaches the meninges through the brain micro vessels and adheres to the endothelial cells of the vasculature. The Tfp then induces the activation of the β2-adrenergic receptor, which in turn activates β-arrstins, leading to the formation of cortical plaques within the cell cytoplasm and the recruitment of actin and the Par6/Par3/PKCζ polarity complex, resulting in the further recruitment of adherens junction proteins. This cytoplasmic recruitment of actin, the Par6/Par3/PKCζ polarity complex, and local adherens junction proteins ultimately alters the intercellular junctions within the endothelial monolayer, causing the formation of gaps between adjacent endothelial cells and subsequent endothelial leakage (Lecuyer et al. 2012, Miller et al. 2012).

Invasion into the meninges and cerebrospinal fluid within the subarachnoid space is accompanied by acute inflammation due to recruitment of inflammatory monocytes and polymorphonuclear neutrophils, and the subsequent secretion of inflammatory cytokines Interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha (TNF-α) (Girardin et al. 1988, Waage et al. 1989, van Deuren et al. 1995, Miller et al. 2012). This inflammatory response then leads to cerebral oedema, raised intracranial pressure, and possibly cerebral herniation and death. Early recognition and treatment is essential for ensuring the best possible outcome, with the mortality rate for meningococcal septicaemia and meningitis approximately 5-20%, with approximately 80% mortality in untreated cases. Non-fatal cases of meningitis or septicaemia are also associated with significant
impairment and sequelae, including but not limited to, permanent hearing loss, mental retardation, loss of limbs, and chronic pain (Stephens et al. 2007, Pace et al. 2012).

1.1.2 Treatment of Neisseria meningitidis infections
The current suggested treatment is for *N. meningitidis* infections intravenous administration of ceftriaxone or cefotaxime to quickly eliminate infection, and management of inflammation, intracranial pressure, coagulopathy, and other associated symptoms (van de Beek et al. 2012). Historically, penicillin and rifampicin have been used in the treatment of *N. meningitidis*, however increasing incidence of penicillin resistance has indicated the need to shift to extended-spectrum cephalosporins as frontline treatments (Bertrand et al. 2012, Klena et al. 2012).

1.2 Neisseria gonorrhoeae
Following chlamydia, gonorrhoea is the second most commonly reported sexually transmitted infection in Australia, with approximately 13,500 new cases reported in 2012 (Lahra 2013), however the actual number of new cases may be higher due to asymptomatic infection. An obligate intracellular pathogen, *Neisseria gonorrhoeae* most commonly colonises the mucosal surfaces of the male and female genital tracts, in particular the ectocervical and endocervical epithelium in women (Edwards et al. 2000), and the urethral epithelium in men (Apicella et al. 1996). Urethral infection in men commonly presents as painful urination and purulent discharge, however inflammation of the prostate or testicles may be present in some cases, with inflammation of the epididymis potentially leading to sterility. Infection in women may be accompanied by cervicitis/urethritis, painful urination, vaginal discharge and abnormalities in menstrual cycle, however it is estimated that up to 80% of infected women may not present with noticeable symptoms, which can lead to prolonged infection and greater risk of sequelae.
(Sparling et al. 2000, Fuchs et al. 2014). Prolonged or untreated infections in women can cause two major complications; pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI). PID is a chronic inflammatory disease of the uterus, fallopian tubes, and ovaries, estimated to occur in 10% of women. Prolonged inflammation of these tissues leads to abnormal scar formation and adhesion to nearby tissues, causing constriction of the fallopian cells, which can result in ectopic pregnancy and infertility (Munday 2000, Edwards et al. 2004). Following colonisation of the mucosa of the lower urogenital tract, *N. gonorrhoeae* may cross the epithelial layer and enter into the vasculature, spreading to other anatomical sites and resulting in DGI. Such disseminated infections are most commonly associated with arthritis and endocarditis, but can also result in dermatitis, fever, meningitis, and septicaemia. Disseminated *N. gonorrhoeae* infections occur in approximately 2% of gonococcal infections (Rice 2005).

Transmission occurs through direct contact of infected mucosal surfaces. Although this normally occurs through contact of the urogenital tract during sexual intercourse, *N. gonorrhoeae* is capable of colonising other mucosal surfaces, including rectal and pharyngeal epithelia. *N. gonorrhoeae* of the rectum is particularly prevalent in women and men who have sex with men, and can result in rectal bleeding, discharge, tenesmus, and disseminated infection. Pharyngeal infections arise from direct oral contact with infected genital mucosal surfaces or discharge. Although infections of the pharynx are generally asymptomatic, complications can arise due to localized inflammation, disseminated infection and transmission between sexual partners (Newman et al. 2007, Mayor et al. 2012).
Another area of infection is the conjunctiva, resulting in eye pain, discharge, conjunctivitis, and blindness. Conjunctival infections can occur in adults, but are of greatest concern in neonates where transmission occurs through the mother during childbirth, leading to neonatal blindness (Mayor et al. 2012).

Regardless of the site of infection, gonorrheal disease is largely due to upregulation of pro-inflammatory cytokines IL-6, IL-8, and TNF-α, in part due to the host response to gonococcal surface proteins such as the LOS, as well as activation of the inflammation regulators activating transcription factor 3 (ATF3) and nuclear factor kappa beta (NF-κB) following attachment to epithelial cells (Fichorova et al. 2001, Harvey et al. 2002, Dietrich et al. 2011, Calton et al. 2013).

1.2.1 Adherence, invasion and intracellular survival of Neisseria gonorrhoeae

Infection of genital epithelial cells by N. gonorrhoeae is a multi-step process, mediated by a large number of outer membrane proteins that facilitate cellular attachment, invasion, intracellular survival, and exocytosis. Initial attachment to epithelial cells is mediated by N. gonorrhoeae type IV pilus (Tfp), peritrichous fibers composed of polymers of pilin subunits, which bind the host membrane co-factor protein, CD46, or α1β1 or α2β1 integrins (Merz et al. 2000a). The pilus then retracts in an ATP-dependent manner, positioning the cell closer to the bacterium near the cell surface (Wolfgang et al. 1998, Merz et al. 2000b). Further attachment is then established through binding of other outer membrane proteins such as the opacity proteins (Opa), which bind to the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM) or heparin sulfate proteoglycans (HSPG) (Sadarangani et al. 2011), LOS, which adhere to the complement receptor CR3 within the female genital tract and the asialoglycoprotein receptor (ASPG-R) within the male genital tract (Hung et al. 2013), and porins, specifically
PorB.1A protein which has been shown to bind the glycoprotein Gp96 (Rechner et al. 2007). Gonococci attachment initiates a number of signaling cascades, including phosphoinositide 3-kinase (PI3K), phospholipase C (PLC), NF-κB and the mitogen-activated protein kinase (MAPK) Erk, to induce engulfment of the bacterium and prevent apoptosis of the host cell. The pilus also serves to alter calcium flux, firstly by delivery of porins to the host membrane, triggering an influx of calcium into the cytosol. Pilus retraction then initiates release of intracellular calcium stores in a process believed to stimulate exocytosis of host endosomes and lysosomes in order to alter these vesicles and promote intracellular bacterial survival (Ayala et al. 2005, Edwards et al. 2013).

Similar to that observed in N. meningitidis, gonococcal entry into the vasculature or sub-epithelial tissues is facilitated by the formation of cytoplasmic cortical plaques following attachment to the epithelial surface. Gonococcal Tfp and outer membrane proteins induce the formation of cortical plaques rich in epidermal growth factor receptor (EGFR). Formation of these cortical plaques results in alteration of the actin cytoskeleton and adherens junctions proteins E-cadherin and β-catenin, at least partly via EGFR-induced phosphorylation of β-catenin. Once phosphorylated, β-catenin dissociates from the adherens junctions and the actin cytoskeleton, disrupting the junction between epithelial cells and allowing transmigration of N. gonorrhoeae into the sub-epithelial tissues and blood, resulting in disseminated infections (Rodriguez-Tirado et al. 2012, Edwards et al. 2013).

1.2.2 Treatment of Neisseria gonorrhoeae infections

Due in part to high variation with gonococcal antigens, the lack of a polysaccharide capsule and the low mortality rate associated with gonorrhoeal infections, there are no
vaccines available for prevention of *N. gonorrhoeae* infections, thus current means of
treatment and control of *N. gonorrhoeae* are antibiotic therapy. Unfortunately, the
response to this has been the rapid development of antimicrobial resistance.
Historically, treatments involved urethral irrigation with silver nitrate or potassium
permanganate (Christian 1894) or heat treatment of infected areas (Kanner 1932). The
introduction of sulphonamides and penicillin allowed quick and efficient treatment of
infections, however the rapid development of resistance occluded these agents as
treatments. The current treatment of gonorrhoeal infections recommended by the United
States Centre for Disease Control is a combination of the third generation cephalosporin
ceftriaxone and the macrolide azithromycin (Dees et al. 1937, Dunlop 1949, Unemo et
al. 2011).

1.3 Antimicrobial resistance in *Neisseria spp.*

The development and use of antibiotics for the treatment of bacterial infections has been
one of the most significant medical achievements in the past century, however this
advancement has been overshadowed by the emergence of antimicrobial resistance,
with widespread development of bacterial resistance to almost all classes of
antimicrobial agents in clinical use. The variety of bacteria becoming resistant to
antibiotics is increasing, as a consequence the number of deaths from antibiotic resistant
bacteria is also rising (Laxminarayan et al. 2014). Cases of multiple drug resistance in
*N. gonorrhoeae* and *N. meningitidis* strains represent a major public health concern due
to rapidly increasing drug resistance and rapidly diminishing treatment options.

1.3.1 Antimicrobial resistance in *Neisseria gonorrhoeae*

*N. gonorrhoeae* has developed resistance to the sulphonamides, penicillins,
tetracyclines, fluoroquinolones, azithromycin, spectinomycin, and the oral
cephalosporin antibiotics, leaving the injectable extended-spectrum cephalosporin (ESC) ceftriaxone as the last and most viable treatment option. Ceftriaxone is currently in widespread global use, however growing numbers of multi drug resistant (MDR) and extensively drug resistant (XDR) (Table 1.1) gonococcal strains resistant to ceftriaxone have been reported (Lewis 2010, Ohnishi et al. 2011, Unemo et al. 2012a, Unemo et al. 2012b, Whiley et al. 2012). From a public health perspective, this is of particular concern due to extremely high rates of infection, and associated high morbidity and socioeconomic consequences. In 2008, the World Health Organisation (WHO) reported more than 100 million new gonorrhoea cases annually among adults aged 15-49 years, representing a 21% increase since 2005, with a call for public health intervention at the international level made by the WHO in 2012. Following this, the Centre for Disease Control and Prevention in the US listed N. gonorrhoeae in the top 3 of all antibiotic resistant threats in 2013. In Australia, close to 5% of all gonococci isolates now show decreased susceptibility to ceftriaxone, and treatment failures with ceftriaxone have been reported (Chen et al. 2013). The current situation is that gonorrhoea that was treatable with antibiotics for the past 70 years is now a significant public health threat both nationally and globally as strains develop resistance to the last remaining option for first-line empiric treatment (Tapsall 2009a, Tapsall 2009b, Goire et al. 2012). Moreover, history has shown that antibiotic resistance is likely to continue to worsen given the global nature and mechanism of transmission. In this context, there are widespread fears of an era of untreatable N. gonorrhoeae approaching, representing a major public health concern both in Australia and worldwide, as well as an urgent need for the development of new antimicrobials.
Definitions of multiple drug resistant and extensively drug resistant 
*N. gonorrhoeae*

<table>
<thead>
<tr>
<th>Definitions of multiple drug resistant and extensively drug resistant</th>
<th><em>N. gonorrhoeae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple drug resistant (MDR)</td>
<td>Resistant to one of the antibiotic classes listed in category I, and two or more in category II</td>
</tr>
<tr>
<td>Extensively drug resistant (XDR)</td>
<td>Resistant to two or more of the antibiotic classes in category I, and three or more in category II</td>
</tr>
</tbody>
</table>

Antibiotic categories

| Category I (recommended antibiotics) | • Extended-spectrum cephalosporins  
• Spectinomycin |
| Category II (less commonly used antibiotics) | • Penicillins,  
• Fluoroquinolones (ciprofloxacin)  
• Azithromycin  
• Aminoglycosides  
• Carbapenems |
| Category III (superseded antibiotics) | • Rifampicin  
• Chloramphenicol  
• Tetracyclines  
• Co-trimoxazole  
• Erythromycin |

Table 1.1 - Definitions of multiple drug resistant and extensively drug resistant *N. gonorrhoeae*. Definitions as suggested by Tapsall et al. (2009c).

1.3.2 Antimicrobial resistance in *Neisseria meningitidis*

In comparison to *N. gonorrhoeae*, antimicrobial resistance in *N. meningitidis* appears less extensive, presumably due to the lower prevalence of invasive meningococcal disease and availability of numerous vaccines. Yet, increasing numbers of *N. meningitidis* isolates exhibiting resistance to numerous antimicrobials, including penicillins, rifampicin, tetracyclines, and sulphonamides, have been reported worldwide (Oppenheim 1997, Stefanelli et al. 2003, Hedberg et al. 2009, Gorla et al. 2011).

Although much rarer in incidence, multiple cases of resistance to chloramphenicol
(Shultz et al. 2003), fluoroquinolones (such as ciprofloxacin) (Alcala et al. 2004, Mehta et al. 2007, Wu et al. 2009), and azithromycin have been reported (Rodriguez et al. 2005, Lapadula et al. 2009). Furthermore, there has been one report of reduced susceptibility to the extended-spectrum cephalosporins ceftriaxone and ceftaxime in isolates from India (Nicolas 2007). Although antimicrobial resistance in *N. meningitidis* is not as prevalent as that observed in *N. gonorrhoeae*, a trend of increasing resistance is clearly visible. The emergence of increased antimicrobial resistance, combined with the limitations of current vaccines against *N. meningitidis*, indicates a clear need for the development of new antimicrobials. In summary, both *Neisseria gonorrhoeae* and *Neisseria meningitidis* represent a major public health concern, due to rapidly increasing drug resistance and diminishing treatment options.

1.4 **Bacterial fatty acid synthesis**

The type II fatty acid synthesis (FASII) pathway is responsible for bacterial lipid biogenesis and membrane homeostasis, and is essential in many bacteria. The enzymes that catalyse the reactions of this pathway, known as Fab (fatty acid biosynthesis) proteins, are structurally distinct from their mammalian counterparts, and thus represent an attractive target for the discovery of new antimicrobial agents. Bacteria utilise the FASII pathway in order to create a range of fatty acids essential for growth, as well as for the production including components of phospholipid membranes, lipoproteins, and LOS. In the FASII pathway, discrete enzymes catalyse condensation of malonyl-CoA to produce a diverse range of products, including unsaturated fatty acids, branched chain fatty acids, and hydroxyl-fatty acid chains (Parsons et al. 2013).

Importantly, the FASII pathway differs substantially from the type I fatty acid synthesis (FASI) pathway of mammalian cells, which acts as a multifunction protein complex by
a single gene, with the various domains of the complex catalysing the transferase, condensation, reduction and dehydration reactions. Whilst the individual reactions of the FASI and FASII pathways are similar, the structural differences between these two systems provide important targets for drug development, as the pathway is essential to bacterial survival and presents minimal effects to the mammalian host. Whilst some recent debate in the literature has arisen regarding whether some Gram-positive bacteria can overcome FASII inhibitors by obtaining fatty acids exogenously from serum, for example Streptococcus pneumoniae (Brinster et al. 2009, Balemans et al. 2010), there is no dispute in the field regarding the essentiality of the FASII pathway in Gram-negative bacteria, including N. gonorrhoeae, and overall, the FASII pathway has been clearly validated as an antibacterial target (Chang et al. 2013, Wang et al. 2013).

The FASII pathway of Neisseria spp. is comprised of a number of discrete dissociated enzymes, each of which catalyses a single step within the pathway (Figure 1.1). The cycle is initiated by the activation of the acyl carrier protein (ACP) by ACP Synthase, which transfers the phosphopantetheine group arm of co-enzyme A (CoA) to apo-ACP, providing the sulfhydryl that serves as the attachment site for FASII intermediates. The malonyl group from malonyl-CoA is then transferred to the terminal sulfhydryl of the phosphopantetheine group of the activated ACP by FabD. The initial condensation reaction or formation of fatty acid is catalysed by FabH, which condenses the acetyl moiety of acetyl-CoA onto malonyl-ACP forming acetoacetyl-ACP, in effect fusing together a 2-carbon product and a 3-carbon product to form a 4-carbon fatty acid intermediate. The product of this condensation reaction, a β-ketoacyl-ACP, is reduced by FabG to form β-hydroxybutyryl-ACP. In Neisseria spp., FabG is the only enzyme that catalyses this reduction reaction. The dehydratase FabZ then catalyses the removal of a proton to form trans-2-enoyl-acyl-ACP. The second reductase of the FASII
pathway, FabI, catalyses the removal of the C2:C3 double bond of trans-2-enoyl-acyl-ACP, forming saturated butyryl-ACP, a saturated fatty acyl-ACP molecule. The acyl-ACP molecule continues to undergo subsequent elongation cycles, in which the condensation of the acyl-ACP and malonyl-ACP is catalysed by FabF until the required chain length is achieved.

The fatty acyl-ACP produced by the essential FASII pathway has three major fates. Firstly, it can be used by the condensing enzyme FabF to initiate a new round of elongation. Secondly, it can be used by the acyltransferase module to transfer these fatty acids to the cell membrane. Thirdly, it can be diverted as an intermediate for synthesis of other cellular metabolites, for example, Lipid A that is needed to form the hydrophobic anchor of LOS. LOS are a major component of the outer membrane of *Neisseria spp.*, as well as the outer membrane of Gram-negative bacteria. They contain at least four β-hydroxy-fatty acids derived from the FASII pathway, essential structural components that cannot be substituted for by normal fatty acids (Zhang et al. 2008). Together with the fact that enzymes of LOS biosynthesis are specific for acyl-carrier protein (ACP) thioesters, this clearly establishes the essentiality of the FASII pathway in Gram-negative bacteria. Whilst some bacteria encode multiple enzymes for the condensation, reduction and dehydration reactions (FabB, FabL/FabK/FabV, and FabA respectively), *Neisseria spp.* possess no such isozymes, making the FASII pathway an especially attractive target for development of novel antimicrobials.
1.4.1 Acyl carrier protein synthase as a drug target

All reactions of the FASII pathway utilise ACP, either as a substrate, such as in the case of ACP synthase and FabD, or as a substrate-linked shuttling protein, in other FASII reactions. ACP is essential for FASII functionality and bacterial survival, due to poor specificity for CoA-linked or free acyl intermediates. Presumably, this is to distinguish the fate of these intermediates as FASII substrates, from those utilised in fatty acid catabolism. However, ACP itself is unlikely to be a suitable drug target due to the high degree of similarity between bacterial ACPs and the human FASI ACP (Figure 1.2).

Although ACP is required for cellular viability, accumulation of apo-ACP, the inactivated form of ACP, is toxic to *E. coli*, and can result in inhibition of phospholipid synthesis at least partly through inhibition of sn-glycerol-3-phosphate acyltransferase.
ACP synthase, the agent which catalyses the formation of functional halo-ACP from covalent attachment of the 4′-phosphopantetheine moiety onto apo-ACP, provides a potential target for the development of new antimicrobial agents. Additionally inhibitors of AcpS have been reported, with IC₅₀ values of approximately 1 μM against the AcpS of *Bacillus subtilis*, and MIC values of 12.5 – 50 μM for *B. subtilis, Enterococcus faecalis,* and *S. pneumoniae* (Gilbert et al. 2004), further supporting AcpS as a drug target.

AcpS is a member of the phosphopantetheinyl transferase (PPTase) protein superfamily, which all catalyse the transfer of the phosphopantetheine group of CoA to a receptor protein. AcpS is a group 1 PPTase, unlike the monomeric group II PPTases found in animals, group I PPTases consist of approximately 120 amino acids and trimeric quaternary structures. Bacteria may possess multiple PPTases, however the specificity of these enzymes appears to vary between species.

For example, the *E. coli* PPTase EntD is unable to functionally replace AcpS. In contrast, *B. subtilis* encodes an AcpS and the PPTase Sfp, which can catalyse the transfer of the phosphopantetheinyl group of CoA to ACP in the absence of AcpS (Lambalot et al. 1996, Copp et al. 2006). Studies involving AcpS under the control of tetracycline and arabinose promoters have indicated that AcpS is an essential protein, with knockdown of this gene also resulting in growth arrest, suggesting inhibition or knockdown of AcpS ultimately causes growth arrest due to accumulation of apo-ACP. However, De Lay et al. (2006) showed that while overexpression of the *E. coli* PPTase YhhU is unable to fully replace AcpS *in vivo* due to poor expression of YhhU or inefficient activation of apo-ACP, YhhU is able to support very slow colony growth in the absence of AcpS. This result contradicts those previously observed (Takiff et al.
1992, Flugel et al. 2000), which indicate AcpS knockdown mutants are non-viable. If this is the case, antimicrobial agents targeting AcpS may be of little use in *E. coli* or other bacteria that possess other PPTase enzymes capable of catalysing the phosphopantetheinylation of apo-ACP. Based on the results established by De Lay et al. (2006), some bacterial growth appears present when neither AcpS nor YhhU, which have both been placed under the control of a tetracycline promoter, are expressed. Whether this growth is due to residual expression from time cultured in the presence of tetracycline or “leaky” expression has not been addressed.

Whilst multiple PPTase enzymes may limit the effectiveness of AcpS inhibitors in some bacteria, no alternate PPTases have been identified in *N. meningitidis* and *N. gonorrhoeae* (Donadio et al. 2007). This combined with the structural differences between Neisserial AcpS and the group I and group II PPTases found in humans, as well as the identification of inhibitors with low IC\textsubscript{50} values and moderate MIC values, indicates AcpS is a promising drug target.
1.4.2 FabD as a drug target

Malonyl-CoA:ACP transacylase or FabD is ubiquitously expressed as a single isoform within bacteria, highly conserved amongst species, and appears to be an essential protein, with inactivation of the fabD gene shown to result in lethality (Zhang et al.)
1998, Zhang et al. 2007). These characteristics implicate FabD as an ideal drug target for the development of broad spectrum antimicrobials, yet despite this, the validity of FabD as a drug target has not clearly been established.

While reports indicate fabD is an essential gene in E. coli (Zhang et al. 1998), the reasons for this are largely unknown, as FabD does not appear to be essential for branched chain fatty acid synthesis (Oku et al. 2003). While AcpS has a greater specificity for CoA than malonyl-CoA, it is able to catalyse the transfer of the malonyl-phosphopantetheine moiety of malonyl-CoA onto apo-ACP, seemingly eliminating the need for FabD in the synthesis of straight chain fatty acids (McAllister et al. 2006). Additionally, FabH and FabF are able to catalyse the transfer of a fatty acid moiety from CoA onto holo-ACP. The products of these transacylation reactions can then be utilised as substrates for the elongation reactions catalysed by these two enzymes (Tsay et al. 1992, Heath et al. 1995, Borgaro et al. 2011). Therefore, FabD must have a greater role in the FASII pathway, presumably involving the regulation of malonyl-CoA and malonyl-ACP pools, or an essential role outside the FASII pathway, such as in the polyketide synthesis pathways.

Despite the ability of these FASII enzymes to circumvent the role of FabD in vitro, in vivo these functions are unable to support cell growth in the absence of functional FabD. This suggests FabD has a greater role than generating malonyl-ACP for fatty acid synthesis, FabD has been implicated in the synthesis of some polyketides, such as tetracenomycin C (Florova et al. 2002), and the anticancer depsipeptide FK228 (Wesener et al. 2011). Furthermore, accumulation of intracellular malonyl-CoA appears to coincide with inhibition of the FASII pathway (Parsons et al. 2011).
Overexpression of FabF has been shown to cause rapid cell growth arrest and increase intracellular malonyl-CoA pools by approximately 80 fold compared to normal levels. Interestingly, FabF induced toxicity is largely overcome by overexpression of FabD (Subrahmanyam et al. 1998), Parsons et al. (2011) suggest malonyl-CoA serves as a regulator of fatty acid synthesis, and that accumulation of malonyl-CoA may result in a metabolic block. This hypothesis is consistent with the finding that FabD prevents growth arrest induced by FabF overexpression, indicating a possible role of FabD in maintaining malonyl-CoA pools and thus regulation of fatty acid synthesis; however, there remains no direct link between FabD and regulation of the FASII pathway.

It is perhaps this uncertainty that has resulted in little development of FabD inhibitors, with only a small number of molecules identified as inhibitors of FabD to date. The aporphine alkaloid corytuberine was found to have an IC$_{50}$ value of approximately 33 µM against FabD from Helicobacter pylori, with molecular docking simulations identifying three related aporphine alkaloid molecules as potential inhibitors (boldine, dicentrine, and glaucine), however their inhibitory activity has not been reported. Additionally, there have been no reported MIC values for corytuberine, boldine, dicentrine, or glaucine against any bacteria, and as such, the antibacterial activity of these compounds, if any, remains unknown (Liu et al. 2006). Sun et al. (2012) investigated the anti-parasitic activity of corytuberine against Eimeria tenella, a parasite known to cause haemorrhagic Coccidiosis in chickens. They found that corytuberine inhibited E. tenella FabD greater than that of H. pylori, yet no significant inhibition of E. tenella growth was observed, with an MIC$_{50}$ of approximately 650 µM. The reasons for the poor anti-parasitic activity of corytuberine have not yet been determined. While the essentiality of FabD in E. tenella has not been assessed, high concentrations of triclosan (≤ 1000 µM), a FabI inhibitor with potent antibacterial and anti-malarial
activity, are unable to inhibit *E. tenella* growth, despite previous reports that triclosan inhibits *E. tenella* FabI with an IC$_{50}$ of 60 nM. *E. tenella* possess both type I and type II FAS pathways, and while it is suggested that these pathways have different metabolic roles, the failure of these two compounds to inhibit *E. tenella* growth suggests *E. tenella* can exclusively utilise the FASI pathway or import exogenous fatty acids (Lu et al. 2007, Sun et al. 2012).

Shen and co-workers (2010) identified five compounds that they believed strongly inhibit FabD. The antimicrobial activity of these molecules was assessed by antimicrobial disc diffusion susceptibility assays, and while some of these molecules appeared to possess slight antimicrobial activity against *E. coli*, no IC$_{50}$ or MIC values were reported. Due to this lack of data, it is impossible to compare these molecules to corytuberine or other potential inhibitors. The aporphine alkaloid inhibitors or the inhibitors reported by Shen et al. (2010) may prove to be valuable lead molecules for the development of new therapeutic agents, however there is currently no strong data to suggest these molecules possess significant antimicrobial activity.

1.4.3 FabF and FabH as drug targets

FabH catalyses the first condensation reaction of the FASII pathway and is thought to be a major determining factor in regulating the ratio of branched chain and saturated fatty acids within the bacterial membrane. Surprisingly, FabH does not appear to be an essential protein (Yao et al. 2012), however a large number of FabH inhibitors with antibacterial activity have been discovered (Wang et al. 2013). FabH strictly prefers short chain (C2-C4) fatty acyl-CoAs and malonyl-ACP (Choi et al. 2000, Qiu et al. 2005, Gajiwala et al. 2009), while FabF prefers medium to long chain fatty acyl-ACPs, and is capable of catalysing the elongation of both long and short chain fatty acyl
molecules via the decarboxylation of malonyl-ACP to acetyl-ACP. Due to these overlapping substrate specificities, FabF is able to catalyse the initial condensation reaction in the absence of FabH, potentially negating the requirement for FabH, however FabH cannot functionally replace FabF (Alberts et al. 1972, Zhang et al. 2006, Morgan-Kiss et al. 2008).

The reliance of the FASII pathway on FabF has led to the discovery of a number of inhibitors that exhibit high affinity for FabF. These include cerulenin, thiolactomycin (Price et al. 2001), platensimycin (Wang et al. 2006), and platencin (Wang et al. 2007). In comparison to platensimycin, thiolactomycin and cerulenin are both relatively weak inhibitors, with IC\(_{50}\) values of 160 nM, 6 µM, and 20 µM, respectively (Price et al. 2001). Perhaps most interesting is the development of platencin, which targets both FabF and FabH. While platencin and platensimycin share a similar chemical structure, platencin exhibits approximately 4 fold greater affinity for FabH (IC\(_{50}\) = 16.2 µM) than platensimycin, yet at the cost of an IC\(_{50}\) value almost 6 fold lower (0.113 µM) than that of its relative (Wang et al. 2007).

Both platensimycin and platencin exhibit low MIC values against the Gram-positive bacteria *Staphylococcus aureus, E. faecalis, and S. pneumoniae*, with the highest MIC value for platensimycin approximately 4.5 µM, and 8 µM for platencin. Furthermore, both molecules showed very little toxicity when applied to HeLa MMT cells (Wang et al. 2006, Wang et al. 2007). These results establish FabF as an excellent target to combat emerging drug resistance.

In contrast, both platensimycin and platencin display very high MIC values (>140 µM) against wild-type *E. coli* (Wang et al. 2006, Wang et al. 2007), with mutation of the
AcrAB–TolC efflux pump shown to significantly enhance the antimicrobial activity of these two molecules against *E. coli*, suggesting that the poor effectiveness of these inhibitors in Gram negative bacteria is largely due to antimicrobial efflux. Whilst *N. meningitidis* and *N. gonorrhoeae* do possess antimicrobial efflux pumps, neither possess the AcrAB–TolC efflux pump found in *E. coli* (Rouquette-Loughlin et al. 2003, Kamal et al. 2007, Kamal et al. 2010), and as such the antimicrobial activity of these inhibitors against *Neisseria spp.* may be more similar to that observed in Gram positive bacteria.

### 1.4.4 FabG and FabI as drug targets

Individually, both FabG and FabI represent excellent antibacterial targets, with several inhibitors already in clinical trials or current use (Maity et al. 2010, Escaich et al. 2011, Chang et al. 2013, Cummings et al. 2013, Kaplan et al. 2013, Zheng et al. 2013, Kim et al. 2014). FabI catalyses the final and rate limiting step of the FASII pathway, and has therefore been the most heavily targeted in drug discovery programs. It is the main target for triclosan, a broad-spectrum antibacterial agent used across a wide range of consumer products (Maity et al. 2010, Mehboob et al. 2010, Saleh et al. 2011, Singh et al. 2011). Significantly, rational structure-based drug design has been used successfully to generate new inhibitors exhibiting strong anti-staphylococcal activities, particularly in MDR *S. aureus*. MUT056399, a triclosan derivative which has potent anti-staphylococcal activity, is currently undergoing clinical trials and was found to be very efficacious *in vivo* in protecting mice against meticillin and vancomycin resistant strains of *S. aureus* (Escaich et al. 2011). AFN-1252, another novel compound targeting FabI, is a potent inhibitor exhibiting targeted activity against *Staphylococcus spp.* It is in Phase II clinical trials for resistant *S. aureus*, and has been developed in both oral and intravenous formulations, with excellent pharmacokinetics, pharmacodynamics, and
efficacy in murine infection models (Banevicius et al. 2013, Kaplan et al. 2013, Tsuji et al. 2013). Other inhibitors of FabI include CG400549, diazepine analogues, Spiro-naphthyridinone piperidines, and isoniazid, the latter a target of InhA (FabI homologue in *Mycobacterium*) and the current drug of choice for front line treatment of tuberculosis (Rawat et al. 2003, Vilcheze et al. 2006). FabG, the second enzyme in the FASII elongation pathway, is ubiquitously expressed and highly conserved in bacteria. Chlorogenic acid and structurally similar catechin compounds have been shown to inhibit FabG in a concentration-dependent manner, the inhibitor FG01 also exhibits potent inhibitory activity against *Pseudomonas aeruginosa* FabG, with an IC$_{50}$ value of 0.02 µM (Li et al. 2006a, Karioti et al. 2007). Together with other publications of FabG and FabI inhibitors, this clearly demonstrates that enzymes of the FASII pathway are valid antibacterial targets.

Whilst low nanomolar inhibitors with selective activity against various bacterial FASII enzymes have entered clinical trials for a range of drug resistant pathogens and clearly validates these enzymes as drug targets, drugs with single targets are prone to mutational resistance, with FabI-specific inhibitors being reported to develop resistance in bacteria through mutations altering the drug-binding site (Yao et al. 2013). Triclosan resistant bacteria and isoniazid resistant *M. tuberculosis* have been reported, due to point mutations in their FabI genes (Webber et al. 2008, Copitch et al. 2010). Resistance to AFN-1252 and CG400549 due to single amino acid substitutions have also been reported (Park et al. 2007, Yao et al. 2013). This emphasises that ideally, antibiotics should bind to multiple targets in order to reduce the likelihood of resistance through mutation.
1.5 Epigallocatechin gallate and related compounds inhibit the FASII reductases FabG and FabI

Screening of large chemical libraries against enzymes of the FASII pathway has been undertaken by several groups and pharmaceutical companies to identify new inhibitors of the FASII enzymes (Zhang et al. 2004, Wang et al. 2006, Wang et al. 2013). Catechin gallate, gallocatechin gallate, and epigallocatechin gallate (EGCG) have been shown to inhibit both FabG and FabI, with IC$_{50}$ values in the low micromolar range (Zhang et al. 2004, Tasdemir et al. 2006), whilst catechin and gallocatechin exhibit no inhibitory activity against FabG or FabI (Table 1.2). EGCG is consistently reported as one of the most potent inhibitors of all the catechins. The galloyl moiety of EGCG has been shown to be essential, but not sufficient, for inhibitory activity (Tasdemir et al. 2006). The mechanism of inhibition in both enzymes has been proposed to occur by EGCG blocking the NADH co-factor binding site (Zhang et al. 2004, Tasdemir et al. 2006). The binding site is also distinct from triclosan and other known FabI inhibitors, providing an exciting opportunity to develop and test new EGCG based inhibitors.

<table>
<thead>
<tr>
<th>Catechin</th>
<th>FabG</th>
<th>FabI</th>
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<tbody>
<tr>
<td>Catechin (1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gallocatechin (2)</td>
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</tr>
<tr>
<td>Gallocatechin gallate (4)</td>
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<td>5</td>
</tr>
<tr>
<td>Epigallocatechin gallate (5)</td>
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<td>15</td>
</tr>
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</table>

Table 1.2 - IC$_{50}$ values (µM) for EGCG derivatives against FabG and FabI (Zhang et al. 2004).
Many Gram-negative bacteria possess resistance to plant polyphenols, including EGCG, due to the AcrAB–TolC efflux pump (Tegos et al. 2002, Taguri et al. 2004). 

*N. gonorrhoeae* however does not possess this efflux pump, and while *N. meningitidis* does possess a TolC-like protein, studies indicate this protein is involved in secretion of FrpC toxin and not efflux of antimicrobials. Instead, *N. gonorrhoeae* and *N. meningitidis* are known to utilise the FarA-FarB, MtrC-MtrD-MtrE, MacA-MacB, and NorM efflux pumps to export antimicrobials (Rouquette-Loughlin et al. 2003, Kamal et al. 2007, Kamal et al. 2010). EGCG and a C16 (palmitate)-EGCG derivative exhibit MIC values of 32 and 16 µg/ml respectively against both *N. meningitidis* and *N. gonorrhoeae* (Matsumoto et al. 2012). These MIC values are far lower than that reported for wild type *E. coli* and TolC mutants (Zhang et al. 2004, Matsumoto et al. 2012), indicating these efflux systems have poor affinity for EGCG, and thus *Neisseria spp.* are incapable of rapidly exporting this compound.

Mutations of Neisserial efflux pumps have been linked to increased antimicrobial resistance (Hagman et al. 1995, Mercante et al. 2012, Golparian et al. 2014); however, there appears to be no reports of mutations conferring resistance to EGCG or related catechins.

**1.6 Summary**

Antibiotic resistance represents a serious public health concern, threatening the loss of effective treatment options for infectious diseases, and undermining many of the most recent advances in medicine, including organ transplants, cancer therapy, and a range of chronic diseases.
*N. gonorrhoeae* infections can result in infertility in men and women, as well as involuntary loss of life due to ectopic pregnancy, neonatal blindness, and disseminated infections associated with arthritis and endocarditis. *N. meningitidis* infections can cause meningitis and septicaemia, which result in coma or death within as little as 12 hours, with roughly 5-20% of cases resulting in fatality. Non-fatal meningitis or septicaemia cases are also associated with significant impairment, including permanent hearing loss, mental retardation, and loss of limbs. Both *N. gonorrhoeae* and *N. meningitidis* represent a major public health concern, due to both the debilitating illnesses that result upon infection, as well as diminishing treatment options due to rapidly increasing drug resistance.

The FASII pathway is a proven target for the development of antimicrobials, with a number of potent molecules already developed. Unfortunately, poor antimicrobial activity against Gram-negative bacteria, in addition to resistance conferred by single point mutations, has limited the effectiveness of these agents.

The FASII enzymes AcpS, FabD, FabH, FabF, FabG, and FabI from *Neisseria meningitidis* FAM18 share between 98-100% sequence identity to MDR and XDR gonococcal strains, thus making them excellent models for the design of new FASII inhibitors to combat the rapidly increasing drug resistance observed in *N. gonorrhoeae* and *N. meningitidis*. Significantly, FabG and FabI, the two reductase enzymes of the FASII pathway, are inhibited by the plant polyphenol EGCG. EGCG has been shown to inhibit FabG and FabI in low micromolar amounts *in vitro*, and exhibit low MIC values against both *N. gonorrhoeae* and *N. meningitidis* (Zhang et al. 2004, Tasdemir et al. 2006).
As such, the specific aims of this work were:

- To determine the three dimensional structures of *Neisseria meningitidis FAM18* FASII enzymes in order to provide a platform for rational structure-based drug design of new antimicrobials agents to target *N. meningitidis* and *N. gonorrhoeae*.

- To determine the structure of EGCG bound to the reductase enzymes FabG and FabI, in an effort to characterise the mechanism of inhibition and provide a lead compound for design of EGCG derivatives with improved inhibitory activity and pharmacological properties.
CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

2.1.1 Molecular biology kits

The QIAGEN QIAprep Spin Miniprep Kit and the QIAGEN QIAquick Gel Extraction Kit were used for preparation of plasmid DNA and agarose gel extraction respectively.

2.1.2 Plasmids

A description of the plasmids and their uses within this study are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRK793: TEV protease</td>
<td>This plasmid encodes the catalytic domain of the Tobacco etch virus (TEV) protease (with S219V mutation), located between a maltose binding protein (MBP) gene and a 6xHis affinity tag. The expressed TEV protease fusion protein cleaves itself from the MBP domain, leaving a catalytic TEV protease with an N-terminal His-tag.</td>
</tr>
<tr>
<td>pMCSG21</td>
<td>This plasmid contains a T7 promoter, N-terminal 6xHis affinity tag, and TEV protease cleavage site upstream of a ligation independent cloning site. A spectinomycin resistance gene allows selection of transformants.</td>
</tr>
<tr>
<td>pMCSG21:Target gene</td>
<td>As above with gene encoding target FASII enzyme cloned into the ligation independent cloning site, the resulting fusion protein contains a N-terminal 6xHis affinity tag and TEV protease cleavage. Three additional residues remain following removal of affinity tag, Serine, Asparagine, and Alanine. Genes encoding the following proteins were cloned: AcpS (YP_975651.1), FabD (YP_974419.1), FabF (YP_974338.1), FabG (YP_974416.1), FabH (YP_974421.1), and FabI (YP_975773.1).</td>
</tr>
</tbody>
</table>
2.1.3 Oligonucleotides

Oligonucleotides used for DNA amplification and sequencing are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcpS_FWD</td>
<td>TACTTCCATCAATGCCATGATTTACGGCATCGGCACAG</td>
</tr>
<tr>
<td>AcpS_REV</td>
<td>TTATCCACTTCCAATGTTATTATTTTTTCGGCAACGACAACGC</td>
</tr>
<tr>
<td>FabD_FWD</td>
<td>TACTTCCATCAATGCCATGTCTTTTGCCTTCTTTTTTCCCG</td>
</tr>
<tr>
<td>FabD_REV</td>
<td>TTATCCACTTCCAATGTTATCAGTGCCTTTCGATAAAGGC</td>
</tr>
<tr>
<td>FabF_FWD</td>
<td>TACTTCCATCAATGCCATGAGTCAGAGAAGAGTAGTCATTACAG</td>
</tr>
<tr>
<td>FabF_REV</td>
<td>TTATCCACTTCCAATGTTATCAGTGCGCTTCGATAAAGAC</td>
</tr>
<tr>
<td>FabG_FWD</td>
<td>TACTTCCATCAATGCCATGACACAAGATTTAAGCGGC</td>
</tr>
<tr>
<td>FabG_REV</td>
<td>TTATCCACTTCCAATGTTATCAGGGCATCACATGCCG</td>
</tr>
<tr>
<td>FabH_FWD</td>
<td>TACTTCCATCAATGCCATGCGATGATGCCAAAAATTTCGGCG</td>
</tr>
<tr>
<td>FabH_REV</td>
<td>TTATCCACTTCCAATGTTATCAGATATTGCAACAGCACCGCC</td>
</tr>
<tr>
<td>FabI_FWD</td>
<td>TACTTCCATCAATGCCATGCGTTTCTGCAAGGCAAAAAATTCGGC</td>
</tr>
<tr>
<td>FabI_REV</td>
<td>TTATCCACTTCCAATGTTATTATCCCTCGGTGCTCAAGCATGATG</td>
</tr>
</tbody>
</table>

2.1.4 Bacterial strains

Strains of *E. coli* used during this study for plasmid amplification and protein expression are listed in Table 2.3.
Table 2.3 - Bacterial strains used in this study and their genotype

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74, recA1, araD139, Δ(ara leu)7697, galU, galK, rpsL (StrR), endA, nupG</td>
</tr>
<tr>
<td>BL21(DE3)pLysS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F-, ompT hsdSB(rB–mB–), gal, dcm (DE3) pLysS (Cam&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells used for routine transformation and DNA amplification; <sup>b</sup>Cells used for protein expression

2.1.5 Solutions and culture media

All solutions and culture media were prepared with sterile reverse osmosis/deionised water. Culture media and solutions used for the preparation of culture media were sterilised by autoclaving for 20 min at 121 °C. All buffers used for protein purification (His/S200 buffers) were filter sterilised (0.22 µm), and collected into sterilised 1 L Schott bottles. The composition of solutions for DNA manipulations, cell culture media and solutions, protein purification and visualisation are listed below in Tables 2.4, 2.5 and 2.6.

Table 2.4 - Solutions used for DNA gel electrophoresis

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE buffer (50x)</td>
<td>2 M Tris, 5.7% glacial acetic acid, 50 mM EDTA</td>
</tr>
<tr>
<td>DNA loading dye</td>
<td>6x DNA Loading Dye (Fermentas)</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>10 mg/mL in water</td>
</tr>
<tr>
<td>DNA Ladder</td>
<td>GeneRuler DNA ladder 100-10,000bp (Fermentas)</td>
</tr>
</tbody>
</table>
### Table 2.5 - Cell culture media and solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB) Broth</td>
<td>10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl</td>
</tr>
<tr>
<td>LB agar</td>
<td>10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar</td>
</tr>
<tr>
<td>NPS (20x)</td>
<td>0.5 M (NH₄)₂SO₄, 1 M KH₂PO₄, 1 M Na₂HPO₄</td>
</tr>
<tr>
<td>5052 (50x)</td>
<td>25% glycerol, 2.5% glucose, 10% α-lactose</td>
</tr>
<tr>
<td>Auto-induction media</td>
<td>10 g/L tryptone, 5 g/L yeast extract, 1x NPS, 1x 5052, 1 mM MgSO₄</td>
</tr>
</tbody>
</table>

### Table 2.6 - Protein purification, visualisation and crystallisation solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie blue stain</td>
<td>0.2% Coomassie brilliant blue R-250, 40% ethanol, 10% glacial acetic acid</td>
</tr>
<tr>
<td>Destain buffer</td>
<td>40% ethanol, 10% glacial acetic acid</td>
</tr>
<tr>
<td>SDS-PAGE sample buffer</td>
<td>Novex 2x Tris-Glycine (Life Technologies)</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>1x MES buffer (Life Technologies)</td>
</tr>
<tr>
<td>His buffer A</td>
<td>50 mM phosphate buffer (pH 8.0) 300 mM NaCl, 20 mM imidazole</td>
</tr>
<tr>
<td>His buffer B</td>
<td>50 mM phosphate buffer (pH 8.0), 300 mM NaCl, 500 mM imidazole</td>
</tr>
<tr>
<td>His (TEV) buffer A</td>
<td>50 mM phosphate buffer (pH 8.0), 500 mM NaCl, 30 mM imidazole, 10% glycerol</td>
</tr>
<tr>
<td>His (TEV) buffer B</td>
<td>50 mM phosphate buffer (pH 8.0), 500 mM NaCl, 500 mM imidazole, 10% glycerol</td>
</tr>
<tr>
<td>S200 buffer</td>
<td>50 mM Tris (pH 8.0), 125 mM NaCl</td>
</tr>
<tr>
<td>S200 (AcpS) buffer</td>
<td>50 mM Tris (pH 8.0), 350 mM NaCl, 10% glycerol</td>
</tr>
<tr>
<td>Tacsimate substitute</td>
<td>300 mM ammonium tartrate dibasic, 250 mM citric acid, 1.2 M malonic acid, 400 mM sodium acetate trihydrate, and 500 mM sodium formate</td>
</tr>
</tbody>
</table>
2.1.6 Co-factors and inhibitors

All co-factors and inhibitors were purchased from Sigma–Aldrich. Stock solutions of co-factors NAD/H, NADP/H, and CoA were dissolved in S200 buffer. NAD/H and NADP/H solutions were prepared at a concentration of 100 mM, and CoA was prepared at a concentration of 20 mM. EGCG was dissolved in 100% DMSO, and the concentration was determined by spectrophotometry at 270 nm using the molar extinction coefficient 11920 /M/cm. All co-factors and inhibitors were stored at -80 °C.

2.2 Methods

General methods

2.2.1 Preparation of chemically competent *Escherichia coli*

Chemically competent *Escherichia coli* strains were prepared via treatment with cold MgCl₂ and CaCl₂. Ten microlitres of *E. coli* Top10 or BL21(DE3)pLysS cell suspension (Life Technologies) was spread onto LB agar plates and incubated overnight at 37 °C. A starter culture was then prepared by inoculating 5 mL of LB broth with a single colony from the above plate, and incubated overnight at 37 °C, shaking at 220 rpm. The following day, 100 mL of LB broth in a 250 mL baffled flask was inoculated with 100 µL of starter culture. Cells were cultured at 30 °C, shaking at 90 rpm until an optical density at 600 nm (OD₆₀₀) of 0.4 – 0.6 was reached. Cells were then harvested by centrifugation at 3000 x g for 10 min at 4 °C, resuspended in 25 mL of ice cold 100 mM MgCl₂ and incubated for 10 min on ice. After incubation, the cells were pelleted by centrifugation, resuspended in 25 mL of ice cold 100 mM CaCl₂ and incubated for 30 min on ice. After incubation, the cells were re-harvested and resuspended in 5 mL of 100 mM CaCl₂ and 20% glycerol before being divided into
200 µL aliquots, flash frozen in liquid nitrogen and then stored at -80 °C. Competency and antibiotic sensitivity of competent cells was assessed before use.

2.2.2 Transformation of competent *E. coli* cells

Transformation of chemically competent *E. coli* cells was performed via the heat shock method. One microlitre of plasmid DNA was added to 50 µL of competent bacterial cells and incubated on ice for 30 min. Cells were then incubated at 42 °C for 45 s, and placed on ice for a further 2 min. Two hundred microlitres of room temperature LB broth (Table 2.5) was added to the cells before incubation for 45 – 60 min at 37 °C, shaking at 220 rpm. Transformants were screened by plating 50 µL of transformation culture on LB plates containing 100 µg/mL of spectinomycin and incubating overnight at 37 °C.

DNA methods

2.2.3 Plasmid preparation

The QIAprep Spin Miniprep Kit was used to obtain and purify plasmid DNA. Plasmid DNA was amplified by culturing a single colony of previously transformed Top10 *E. coli* cells in 5 mL of LB broth (Table 2.5) containing the desired antibiotic (Table 2.1) overnight at 37 °C with shaking at 220 rpm. Bacterial cells were harvested by centrifugation at 3000 x g for 5 min, then resuspended in 250 µL of buffer P1 (containing RNase A). *E. coli* cells were lysed using 250 µL of buffer P2 (containing LyseBlue reagent), and genomic DNA was precipitated by the addition of 350 µL of buffer N3. Genomic DNA and cell debris was pelleted by centrifugation at 14000 x g for 15 min. Supernatant was loaded onto the QIAprep spin column and passed through the column by centrifugation for 1 min at 4500 x g. Plasmid DNA was then washed
with 500 µL of buffer PB and 2x 750 µL of buffer PE (containing ethanol) before being eluted in 50 µL of buffer EB.

2.2.4 DNA separation and visualisation
DNA fragments were separated using 1% agarose gels containing 0.01 mg of ethidium bromide per 50 mL of agarose gel, submersed in 1x TAE buffer (Table 2.3) within a horizontal slab apparatus. DNA samples were prepared by the addition of 2 µL of 6x DNA loading dye (Table 2.3) per 10 µL of sample. Samples were run alongside Fermentas GeneRuler DNA ladder for comparison of molecular weight. Electrophoresis was performed at 70 V for approximately 2 h. Following electrophoresis, samples were illuminated and photographed under UV light.

2.2.5 Ligation independent cloning
The genes encoding Neisseria meningitidis AcpS, FabD, FabF, FabG, FabH, and FabI (accession numbers: YP_975651.1, YP_974419.1, YP_974338.1, YP_974416.1, YP_974421.1, and YP_975773.1 respectively) were cloned into the expression vector pMCSG21 by ligation independent cloning, using a similar protocol to Eschenfeldt et al. (2009). Cloned constructs contained full-length proteins fused to a Tobacco etch virus (TEV) protease cleavage site and an N-terminal 6-histidine tag.

2.2.6 Amplification of target genes by polymerase chain reaction (PCR)
Target genes were amplified from genomic DNA (American Type Culture Collection, ATCC), with forward and reverse primers incorporating additional nucleotides to the 5’ and 3’ ends of the target DNA. 5’ TACTTCCAATCCAATGCC---3’ is added to the sense primer, and 5’ TTATCCACTTCCAATGT---3’ to the antisense primer (--- indicates target DNA). These additional nucleotides form overhangs complimentary to
the ligation site of pMSCG21 following incubation with T4 DNA polymerase. All reactions were performed using HotStarTaq Master Mix (Qiagen). The components of PCR reactions and thermal cycling details are detailed below in Tables 2.7 and 2.8.

### Table 2.7 - HotStarTaq DNA PCR components

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>20.5 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>HotStarTaq master mix</td>
<td>25 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.5 µL</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.5 µL</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>1.5 µL</td>
<td>&lt;1 µg/50 µL</td>
</tr>
</tbody>
</table>

### Table 2.8 - HotStarTaq DNA polymerase thermal cycles

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial activation</td>
<td>95</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>94</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>58</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5. Repeat steps 2-4</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>6. Final extension</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>7. Hold</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

#### 2.2.7 Linearisation of pMCSG21 vector

Vector DNA was linearised with SspI. Approximately 5 µg of vector DNA was incubated with 1x SspI buffer (Promega) and 40 U of SspI (HC, GQ; Promega), at 37 °C for 2 h (Table 2.9). For size comparison, pMCSG21 was also digested with NdeI at 37 °C for 2 h as per Table 2.10.
<table>
<thead>
<tr>
<th>Table 2.9 - SspI digestion of pMCSG21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>Vector DNA</td>
</tr>
<tr>
<td>SspI 10x reaction buffer</td>
</tr>
<tr>
<td>SspI</td>
</tr>
<tr>
<td>Sterile H₂O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table - 2.10 NdeI digestion of pMCSG21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>Vector DNA</td>
</tr>
<tr>
<td>NdeI 10x reaction buffer</td>
</tr>
<tr>
<td>NdeI</td>
</tr>
<tr>
<td>Sterile H₂O</td>
</tr>
</tbody>
</table>

2.2.8 Purification of PCR products and linearised vector DNA

PCR products and linearised vector DNA were purified using the Qiagen QIAquick Gel Extraction Kit as per manufacturer’s instructions. Amplicons and vector DNA were separated by agarose gel electrophoresis (section 2.2.4), and excised from the agarose. Agarose containing the DNA fragment of interest was dissolved by the addition of three volumes of buffer QG to one volume of agarose gel, heating at 50 °C, and vortexing every 2 min until dissolved. One gel volume of isopropanol was added to the sample before being loaded onto the QIAquick column and passed through the column by centrifugation for 1 min at approximately 14000 x g. The flowthrough was discarded and target DNA was washed with 2x 500 µL of buffer QG and 2x 750 µL of buffer PE (containing ethanol) before being eluted in 30 µL of buffer EB. Following purification of target DNA, the concentration of DNA was determined by spectrophotometry at 260 nm.
2.2.9 Treatment with T4 DNA polymerase

Complimentary 15 base pair overhangs were created on linearised vector DNA and PCR products by treatment with T4 DNA polymerase (LIC qualified; Novagen) in the presence of either dGTP or dCTP. Linearised vector DNA (section 2.2.7) was incubated with T4 DNA polymerase and dGTP (Promega). The 3’-5’ exonuclease activity of T4 DNA polymerase hydrolyses nucleotides from the 3’ ends of the vector until reaching a guanosine residue, creating a 15 base pair 5’ overhang. Similarly, purified PCR product (section 2.2.8) was incubated with T4 DNA polymerase and dCTP (Promega). The exonuclease activity of T4 DNA polymerase hydrolyses nucleotides from extensions added to target DNA by the LIC specific primers (section 2.2.6; Table 2.2), creating a 15 base pair overhang complimentary to the T4 DNA polymerase treated vector. The components of the T4 treatment reactions are detailed below (Table 2.13, Table 2.14).

Samples were incubated at 22 °C for 30 min, followed by 20 min at 75 °C.

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified vector DNA</td>
<td>As per concentration (200 ng)</td>
<td>5 ng/µL</td>
</tr>
<tr>
<td>10x T4 DNA polymerase reaction buffer</td>
<td>4 µL</td>
<td>1x</td>
</tr>
<tr>
<td>dCTP (100 mM)</td>
<td>1 µL</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>DTT (100 mM)</td>
<td>2 µL</td>
<td>5 mM</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>1.5 µL</td>
<td>~4U</td>
</tr>
<tr>
<td>Sterile H₂O</td>
<td>Made up to 40 µL</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Following T4 DNA polymerase treatment, PCR product was combined with 7.5 ng (1.5 µL) of vector DNA at a 3:1 ratio of insert to vector, and incubated on ice for 30 min to allow T4 treated fragments to anneal. Top10 *E. coli* cells were then transformed using 2.5 µL of reaction mixture as described in section 2.2.2.

### 2.2.10 Confirmation of insert by colony PCR

To confirm the presence of the target gene within the pMCSG21 expression vector, transformants were screened via PCR using HotStarTaq Master Mix (Qiagen). Using a sterile pipette tip, approximately ¼ of a single colony was added to the PCR reaction mixture outlined in Table 2.13, and gently resuspended. All reactions were performed using primers listed in Table 2.2, and reaction cycles as per Table 2.8. Negative controls were prepared as above, using non-transformed *E. coli* Top10 cells as template DNA.

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified PCR product</td>
<td>As per concentration (80 ng)</td>
<td>1 ng/µL</td>
</tr>
<tr>
<td>10x T4 DNA polymerase reaction buffer</td>
<td>4 µL</td>
<td>1x</td>
</tr>
<tr>
<td>dCTP (100 mM)</td>
<td>1 µL</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>DTT (100 mM)</td>
<td>2 µL</td>
<td>5 mM</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>1.5 µL</td>
<td>~4 U</td>
</tr>
<tr>
<td>Sterile H_2O</td>
<td>Made up to 40 µL</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 2.13 - Colony PCR components

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FWD primer (10 µM)</td>
<td>0.75 µL</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>REV primer (10 µM)</td>
<td>0.75 µL</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>HotStarTaq Master Mix</td>
<td>12.5 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>Sterile H2O</td>
<td>10.5 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>Template DNA</td>
<td>¼ of a colony</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2.2.11 DNA sequencing

To confirm that the target gene was incorporated into the vector and that no mutations or frame shift errors had occurred, purified recombinant plasmids obtained via LIC were confirmed by sequencing at the Australian Genome Research Facility (AGRF). Typically, 600 – 1000 nucleotide bases were obtained for each sequencing reaction.

Protein methods

2.2.12 Protein expression

All recombinant constructs were expressed in E. coli BL21(DE3)pLysS (Life Technologies) cells using auto-induction media (Studier, 2005). Competent E. coli BL21(DE3)pLysS cells were transformed with recombinant plasmid as per section 2.2.2. Five single colonies were incubated in 5 mL of LB broth (Table 2.5) containing 100 µg/ml spectinomycin overnight at 37 °C, with shaking at 220 rpm. Eight 2 L flasks containing 500 mL of auto-induction media supplemented with spectinomycin (100 µg/ml) and chloramphenicol (34 µg/mL) were inoculated with 1 mL of overnight culture and incubated overnight at 25 °C with shaking at 90 rpm, until an OD600 of 6 –
10 was reached. Cells were harvested by centrifugation for 30 min at approximately 7500 x g, resuspended in His buffer A and stored at -20 °C until use.

### 2.2.13 Protein purification

Resuspended cells were lysed by two repetitive freeze-thaw cycles, and the addition of 20 mg of lysozyme, followed by the addition of 1 mg of DNase I (Sigma-Aldrich) to reduce sample viscosity. Cells were incubated on ice for 30 min and mixed until homogeneous. The cell lysate was clarified by centrifugation for 20 min at approximately 27000 x g. Soluble cell lysate was filtered using a 0.45 µm low protein binding filter (Millipore) and injected onto a 5 mL Ni-Sepharose HisTrap HP (GE Healthcare) column equilibrated with His buffer A. After sample injection, the matrix was washed with 10-15 column volumes of His buffer A to remove unbound protein. His-tagged protein was eluted by applying His buffer B (Table 2.6) to the matrix, using a linear increase in imidazole concentration from 20 mM to a final concentration of 500 mM (Figure 2.1). Fractions containing the desired recombinant protein were pooled and incubated overnight at 4 °C with ~800 µg of TEV protease to remove the 6xHis tag. After cleavage of the 6xHis tag, the sample was applied to a pre-equilibrated Superdex 200 26/60 size exclusion column (S200, GE Healthcare) and protein was eluted in S200 buffer. Protein was concentrated using an Amicon ultracentrifugal device (Millipore) with a 10 kDa molecular weight cut off, aliquoted and stored at -80 °C. The concentration of purified Fab proteins was then assessed by spectrophotometry at 280 nm in conjunction with extinction coefficients calculated using the ExPasy ProtParam tool (Wilkins et al. 1999) and the amino acid sequences of Fab proteins.
2.2.14 Production of TEV protease

Plasmid DNA encoding TEV protease (Table 2.1) was transformed into BL21(DE3)pLysS cells. Five colonies were cultured overnight in 5mL of LB broth containing 100 µg/mL ampicillin at 37 °C, shaking at 220rpm. The following day, 2x 500 mL of LB broth containing 100 µg/mL ampicillin was inoculated using 2 mL of overnight culture. Culture was grown at 37 °C with shaking at 90 rpm until an OD600 of 0.4 was reached. The culture was then incubated for 2 h at 20 °C, before the addition of 500 µL of 1 M isopropyl β-D-1-thiogalactopyranoside (Promega). Cells were incubated for a further 20 h at 20 °C, then harvested by centrifugation for 30 min at approximately 7500 x g. Harvested cells were resuspended in His (TEV) Buffer A (Table 2.6), and frozen overnight at -20 °C. Cells were lysed by freeze-thaw cycle, the addition of 20 mg of lysozyme, and 1x Fast Break Cell Lysis reagent (Promega). DNase I (Sigma-Aldrich) was added to the sample and incubated on ice for 30 min. Cell debris was removed by centrifugation at 27000 x g for 20 min, the supernatant was then collected and filtered using a 0.45 µm low protein binding filter (Millipore). The soluble cell lysate was injected onto a 5 mL HisTrap HP column (GE Healthcare) equilibrated with His (TEV) buffer A (Table 2.6). After sample injection, the matrix was washed with 15 column volumes of His (TEV) buffer A to remove un-tagged proteins. TEV protease was eluted by applying an increasing gradient of His (TEV) buffer B (Table 2.6) to the column. Fractions containing recombinant TEV protease were pooled, aliquoted and stored at -80 °C. The eluted sample was not purified by size exclusion chromatography or concentrated using Amicon ultracentrifugal device, as the protein rapidly precipitates following elution from the column.
2.2.15 SDS-PAGE analysis

The effectiveness of protein expression and purification (sections 2.2.12 to 2.2.14) was analyzed by SDS-PAGE using samples taken at multiple points throughout protein expression and purification (described in Table 2.16). Samples were separated using precast 4 – 12% polyacrylamide/Bis-Tris gels (Life Technologies) and 1x MES running buffer. Whole cell, soluble cell lysate, and column flow through samples were prepared using a 1:10 ratio of sample to 2x SDS sample buffer (Life Technologies). All other samples were prepared at a 1:1 ratio. Samples were heated for 2 min at 80 °C, followed by vortexing for 1 min and centrifugation at 14000 x g for 1 min. Supernatant was loaded into the wells of the polyacrylamide gel, alongside a Precision Plus Protein Standards (Bio-Rad) molecular weight marker (see Appendix 2). Electrophoresis was performed at 150 V for approximately 35 min. Gels were stained in Coomassie blue solution for approximately 20 min and destained using successive changes of destain solution over a period of approximately 1 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>Taken after cell lysis and treatment with DNase I, prior to removing cell debris by centrifugation.</td>
</tr>
<tr>
<td>Soluble cell lysate</td>
<td>Taken after centrifugation of the whole cell extract to remove cell debris, and filtration by 0.45 μm low protein binding filter.</td>
</tr>
<tr>
<td>Affinity chromatography flowthrough</td>
<td>Taken during washing of the affinity chromatography column following sample injection; contains unbound/non-tagged proteins</td>
</tr>
<tr>
<td>Affinity chromatography elution</td>
<td>Taken from pooled fractions after elution of recombinant protein from the affinity chromatography column.</td>
</tr>
<tr>
<td>Tag removal</td>
<td>Taken after overnight incubation of protein sample with TEV protease.</td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>Taken from pooled fractions collected following size exclusion chromatography; contains recombinant protein.</td>
</tr>
<tr>
<td>Concentrated protein</td>
<td>Contains concentrated protein obtained following affinity chromatography, size exclusion chromatography and concentration of protein using an Amicon ultracentrifugal device.</td>
</tr>
</tbody>
</table>
2.2.16 Enzymatic assays

Activity of *Neisseria meningitidis* FabG and FabI was measured spectrophotometrically by the oxidation of NADPH (in the case of FabG) or NADH (in the case of FabI) at 340 nm similarly to that described previously (Heath et al. 1995, Zhang et al. 2003). The assays were performed using 50 μM substrate (acetoacetyl-CoA for FabG assays, and 2-butenoyl-CoA for FabI assays), 100 μM co-factor, 0.2 μg of recombinant FabI protein and 0.15 μg of recombinant FabG, and 1% DMSO, and made to a final volume of 100 μL with a solution of 125 mM NaCl, 20 mM HEPES pH 7.4 in a 96 well UV-transmissible plate. EGCG was added to the reaction mixture at concentrations of 1 μM, 10 μM, 50 μM, 100 μM, and 250 μM, and incubated for 5 min. The reaction was started by the addition of substrate. A decrease in absorbance at 340 nm was recorded.
over 5 min and readings for the linear portion of the assay used to calculate enzymatic activity. Reactions lacking enzyme with and without EGCG were used as controls. All assays were performed with 1% DMSO to accommodate the solvent in which EGCG was dissolved.

2.2.17 Protein crystallisation

Protein crystallisation was conducted using the hanging drop vapour diffusion technique. Crystallisation was performed in VDX 48-well (500 µL, 22mm diameter) plates (Hampton Research), using hanging drops consisting of 1.5 µL of reservoir solution combined with 1.5 µL of concentrated protein solution on 22 mm diameter cover slips, suspended over 300 µL of reservoir solution. To determine initial crystallisation conditions, sparse matrix crystal screening was performed using concentrated protein samples and commercially available crystallization screens from Hampton Research (Crystal Screen and Crystal Screen II [conditions 1-96], Peg/Ion I & II) and Molecular Dimensions (Proplex I & II, Pact Premier I & II). Initial crystallisation conditions were then optimised by altering salt, buffer and/or precipitant concentrations, pH, and protein concentration, in a stepwise fashion. All crystallisation screens and optimisations were incubated at 23 °C. Prior to data collection, crystals were placed in cryoprotectant (as specified in results chapters) and flash-cooled in liquid nitrogen.

2.2.18 Collection and processing of X-ray diffraction data, and structure determination

All X-ray diffraction data were collected at the Australian Synchrotron on either MX1 or MX2 crystallography beamlines. Collected data were indexed and merged using iMosflm (Battye et al. 2011) or XDS (Kabsch 2010) and scaled with Aimless (Evans et
al. 2013) from the CCP4 suite (Winn et al. 2011). The number of molecules within the asymmetric unit was estimated based on the Matthews coefficient (VM) and predicted molecular weight of the protein (Matthews 1968, Kantardjieff et al. 2003). To determine phases and generate the initial electron density map, molecular replacement was performed in Phaser (McCoy et al. 2007) using the search models detailed in the results chapters. Iterative cycles of model rebuilding with WinCoot (Emsley et al. 2010) were used for manual model building and real space refinement between successive rounds of structure refinement using Refmac5 (Murshudov et al. 2011) and Phenix (Afonine et al. 2012). Any potential metal ions detected within the X-ray crystal structures were validated using WinCoot (Emsley et al. 2010) and the CheckMyMetal (CMM): Metal Binding Site Validation Server (http://csgid.org/csgid/metal_sites/) (Zheng et al. 2014)

2.2.19 Small angle X-ray scattering

Small angle X-ray scattering (SAXS) data were collected at the Australian Synchrotron as previously described (Swarbrick et al. 2014). Serial dilutions of protein solutions ranging from 0.1 – 5 mg/mL were passed through a 1.5 mm quartz capillary at a rate of 4 µl/s. Scattering measurements consisted of ten one second exposures and were performed at 25 °C using an X-ray energy of 12 KeV (Q range 0.01 to 0.5 Å-1) and a Pilatus 1M detector (Dektris). Scattering intensities I(q) for sample and buffer were recorded as a function of scattering vector q. Experimental scattering profiles were calculated using scatterbrain (Australian Synchrotron) after averaging of exposures and background scattering. Theoretical scattering curves of crystallographic structures and ensembles generated by the Proteins Interfaces, Structures, and Assemblies (PISA) server were calculated using CRYSOL (Svergun et al. 1995) and compared to experimentally determined scattering profiles.
CHAPTER 3 - Structural characterisation of the Neisseria FASII enzymes ACP synthase and FabD

3.1 Introduction

Holo-acyl carrier protein synthase, or AcpS, catalyses the activation of apo-acyl carrier protein, a crucial step in fatty acid biosynthesis. The acyl carrier protein (ACP) plays a vital role in shuttling fatty acid intermediates between enzymes of the FASII pathway. To bind these fatty acid intermediates, ACP must first undergo post-translational conversion involving the covalent attachment of the 4′-phosphopantetheine moiety (P-pant) from CoA onto apo-ACP. This post-translational modification of ACP is catalysed by the phosphopantetheinyl transferase (PPTase) holo-acyl carrier protein synthase (AcpS) (Figure 3.1). While ACP is a crucial component of the FASII system, accumulation and overexpression of apo-ACP has been shown to be toxic in E. coli (Keating et al. 1995). The accumulation of apo-ACP in vivo has only been detected following depletion of intracellular CoA, indicating that the conversion of apo-ACP to holo-ACP is strongly favoured in order to avert the toxic effects of apo-ACP accumulation. Theoretically, accumulation of apo-ACP could inhibit FASII enzymes and thus fatty acid synthesis by binding to the same sites as ACP-linked substrates. Yet, Keating et al. (1995) state that low levels of apo-ACP overexpression do not inhibit enzymes of the FASII pathway. Keating et al. (1995) arrived at this conclusion based on analysis of free fatty acid (FFA) content following overexpression of apo-ACP and TesA, a thioesterase able to cleave the fatty acid–ACP thioester bond creating FFAs, which showed intracellular levels of FFAs were increased by overexpression of apo-ACP and TesA. However, overexpression of TesA alone also led to an increase in FFA content, providing an additional variable that may influence fatty acid and lipid
synthesis, thus further *in vitro* analysis needs to be performed to assess the effect of apo-ACP overexpression on enzymes of the FASII pathway.

Despite the uncertainty regarding its intracellular effects, accumulation of apo-ACP has been demonstrated to cause growth arrest (Keating, Carey, & Cronan, 1995). Knockdown of *E. coli* AcpS has been shown to result in growth arrest in at least two studies, suggesting AcpS knockdown mutants are non-viable and that inhibition or knockdown of AcpS ultimately causes growth arrest due to accumulation of apo-ACP (Takiff et al. 1992, Flugel et al. 2000). However, De Lay et al. (2006) claim to have demonstrated that overexpression of the *E. coli* PPTase YhhU is able to support very slow colony growth in the absence of AcpS. The results of De Lay et al. (2006), while contentious, illustrate that antimicrobial agents targeting AcpS may be of little use in bacteria possessing alternate PPTase enzymes.

Fortunately, the limitations faced in using AcpS inhibitors as therapeutic agents do not appear to extend to combating *N. meningitidis* and *N. gonorrhoeae* infections, as no alternate PPTases (homologues of EntD, Sfp or YhhU) have been identified (Donadio et al. 2007). This indicates that the AcpS inhibitors described by Gilbert et al. (2004) and Joseph-McCarthy et al. (2005) that target *B. subtilis* AcpS may exert similar antimicrobial activity against Neisserial species. Furthermore, the predicted structural differences between Neisserial AcpS and the PPTases found in humans, combined with the lack of alternate PPTases in *N. meningitidis* or *N. gonorrhoeae*, and its essential role in fatty acid synthesis, positions AcpS as a promising antibacterial drug target.

Another enzyme that plays a role in the initiation of the FASII pathway is FabD or malonyl-CoA:ACP transacylase, which catalyses the transfer of the three carbon
malonyl groups from malonyl-CoA onto the P-pant arm of holo-ACP (Figure 3.1). The resulting malonyl-ACP is a primary building block of fatty acid synthesis, being utilised by both FabH and FabF to elongate the fatty acid chain.

While the potential of FabD as an antimicrobial drug target is somewhat uncertain, the gene encoding FabD has been identified as essential by a number of groups (Harder et al. 1974, Zhang et al. 1998, Kobayashi et al. 2003, Lee et al. 2009), yet the reason why this enzyme is essential is still largely unknown. As stated earlier, FabD does not appear to be required for the synthesis of either branched or straight chain fatty acids. While the preferred substrate of AcpS appears to be CoA, AcpS has been demonstrated to catalyse the transfer of the malonyl-phosphopantetheine moiety of malonyl-CoA onto apo-ACP, theoretically supplanting the role of FabD (Cox et al. 2002). Apo-ACP has also been found to self acylate by transfer of the malonyl group from malonyl-CoA, forming malonyl-ACP (Misra et al. 2009), and the condensing enzymes FabH and FabF are able to catalyse the transfer of a fatty acid moiety from CoA onto holo-ACP in vitro (Tsay et al. 1992, Heath et al. 1995, Borgaro et al. 2011). Yet, these functions are unable to support cell growth in the absence of functional FabD (Harder et al. 1974, Kobayashi et al. 2003).

FabD has been implicated in the synthesis of polyketides, and maintaining malonyl-CoA pools, and thus regulation of fatty acid synthesis. These potential roles would explain the essentiality of this enzyme, however there remains no direct link between FabD and regulation of the FASII pathway (Florova et al. 2002, Parsons et al. 2011, Wesener et al. 2011).
Only a small number of FabD inhibitors have been identified, with the aporphine alkaloid corytuberine found to have an IC\textsubscript{50} value of approximately 33 µM against FabD from \textit{H. pylori}. The three related aporphine alkaloids, boldine, dicentrine, and glaucine are also suspected to inhibit FabD, however no minimum inhibitory concentration (MIC) values have been reported for corytuberine, boldine, dicentrine, or glaucine against any bacteria, thus the antibacterial activity of these compounds remains unknown (Liu et al. 2006). Sun et al. (2012) showed corytuberine displays no significant inhibition of \textit{E. tenella} growth, with an MIC\textsubscript{50} value of approximately 650 µM, despite showing greater \textit{in vitro} inhibition of \textit{E. tenella} FabD than that of \textit{H. pylori} FabD. However, \textit{E. tenella} possesses both type I and type II FAS pathways and may be able to utilise the FASI pathway or import exogenous fatty acids if FabD is inhibited (Lu et al. 2007, Sun et al. 2012). Shen and co-workers (2010) also identified a number of FabD inhibitors, yet no IC\textsubscript{50} or MIC values were reported.

Due to the potential of AcpS and FabD as antimicrobial targets, as well as the lack of developed inhibitors, we sought to solve the three dimensional structures of these enzymes to aid in the development of new therapeutic agents for the treatment of drug resistant \textit{N. meningitidis} and \textit{N. gonorrhoeae} infections.
3.2 Neisseria ACP synthase

3.2.1 Structure determination

To determine the X-ray crystallographic structure of Neisseria meningitidis AcpS, the gene encoding AcpS synthase (No. YP_975651.1) was amplified from N. meningitidis FAM18 genomic DNA (ATCC catalogue number: 700532D-5) by PCR (as per section 2.2.6) and treated with T4 DNA polymerase in the presence of dCTP to generate extensions complimentary to the LIC site of expression vector pMCSG21. Purified pMCSG21 was linearised by SspI and treated with T4 DNA polymerase in the presence of dGTP to generate the LIC cloning site. The insert encoding N. meningitidis AcpS
(NmAcpS) was incubated with pMCSG21 at a 3:1 ratio of insert to expression vector on ice for 30 min to allow the two products to anneal. Top10 E. coli cells were then transformed using 2.5 µL of reaction mixture as described in section 2.2.9. Spectinomycin resistant colonies were screened by colony PCR to confirm the presence of NmAcpS (section 2.2.10), and recombinant plasmids sequenced (section 2.2.11) to ensure the fidelity of the clone.

Recombinant NmAcpS was solubly over-expressed in E. coli BL21(DE3)pLysS cells using auto-induction media as described in section 2.2.12, and purified using a combination of Ni+ affinity chromatography with a HisTrap HP column followed by size exclusion chromatography using a Superdex 200 26/60 size exclusion column, as per section 2.2.13. Initial attempts to purify NmAcpS resulted in protein precipitation during concentration of the enzyme with an Amicon ultracentrifugal concentrator. To enhance protein solubility, affinity chromatography was performed utilising a buffer with increased NaCl concentration and 10% glycerol. AcpS was successfully purified using these buffers and concentrated to ~35 mg/mL as determined by spectrophotometry using an extinction co-efficient of 9970/M/cm (Figure 3.2).

Initial sparse matrix crystallisation trials were performed by the hanging drop vapour diffusion method using undiluted NmAcpS and the commercial crystallography screens listed in section 2.2.17. Large tetrahedral shaped crystals were observed in several conditions from the Hampton Research crystal screens PEG/ION and PEG/ION 2 after 1 week. Crystals grown in 200 mM sodium citrate tribasic and 20% PEG3350 (Hampton Research PEG/ION condition no. 46) appeared to be of the highest quality by visual inspection and were thus selected for X-ray diffraction. Prior to data collection, crystals were cryoprotected in 25% glycerol and flash cooled in liquid nitrogen.
Figure 3.2 - Expression and purification of *N. meningitidis* AcpS (*NmAcpS*).

*NmAcpS* was purified using a combination of affinity chromatography (A) and size exclusion chromatography (B). Pink line indicates the point of sample injection; blue line represents absorption in mAU; green line indicates the concentration of elution buffer (His buffer B). Protein expression and purification were visualised by SDS-PAGE (C), with samples loaded as follows: protein standards (i), whole cell extract (ii), cell lysate (iii), affinity column flowthrough (iv), affinity column elution (v-vii), cleavage of affinity tag by TEV protease (viii), size exclusion fractions (ix-xii), and concentrated *NmAcpS* (xiii). The single *NmAcpS* crystal used for data collection (D). *NmAcpS* crystals were grown in 200 mM sodium fluoride, 20 % PEG 3350, and 5mM TCEP. Collected diffraction data at 1° (E) and 90° (F) rotation.
X-ray diffraction data were collected at the Australian Synchrotron on the MX2 micro crystallography beamline. A total of 180° of rotation were collected at 0.5° oscillations at a wavelength of 0.9537 Å. Data were integrated and scaled using iMosflm and Aimless to a resolution of 2.00 Å. The large tetrahedral crystals displayed P2\(_1\)2\(_1\)2\(_1\) symmetry with the unit cell parameters \(a = 48.06, b = 90.07, c = 93.05\) Å, \(\alpha = 90, \beta = 90, \gamma = 90°\). Based on a molecular weight of \(~14000\) Da, the crystal was estimated to contain three \(Nm\)AcpS molecules in the asymmetric unit, with a solvent content of 48.73% and a Matthews coefficient of 2.40 Å\(^3\)/Da. This was confirmed by placement of a trimer within the asymmetric unit by molecular replacement with Phaser, using a monomer of \(Vibrio cholerae\) AcpS (PDB:3QMN; Halavaty et al. 2013) as a search model, and an estimated three molecules within the asymmetric unit. Following model building and structure refinement (section 2.2.18), the final R and R-free values were 0.185 and 0.224, respectively (see Table 3.1 for details).

In order to further characterise the substrate binding site of \(Nm\)AcpS, crystals containing bound Co-enzyme A (CoA) were obtained via co-crystallisation, employing the same crystallisation conditions as apo \(Nm\)AcpS, at a 1:10 ratio of \(Nm\)AcpS to CoA. After 3 days, tetrahedral crystals similar to those seen with apo \(Nm\)AcpS were observed. Crystals were cryoprotected and flash cooled as per apo \(Nm\)AcpS crystals, and a total of 180° rotation of X-ray diffraction data were collected at the Australian Synchrotron on the MX2 micro crystallography beamline, at 0.5° oscillations and a wavelength of 0.9537 Å. Data were integrated using XDS and scaled using Aimless to a resolution of 1.75 Å. The crystal displayed the same \(P2_12_12_1\) symmetry as the apo \(Nm\)AcpS crystal, with similar unit cell parameters, \(a = 47.51, b = 92.69, c = 92.00\) Å, \(\alpha = 90, \beta = 90, \gamma = 90°\). As such, the asymmetric unit was expected to contain the same number of AcpS molecules as the apo \(Nm\)AcpS crystal. Three AcpS monomers were placed by
rigid body refinement in Refmac5, using the apo NmAcpS structure as a starting model.

CoA molecules were fitted to the model using a combination of Phenix ligand fit, superposition of other AcpS structures in complex with CoA (*Bacillus subtilis* PDB:1F7L (Parris et al. 2000); *Streptomyces coelicolor* PDB:2WDO, 2WDS (Dall'aglio et al. 2011); *Mycobacterium tuberculosis* PDB:3HQJ (Dym et al. 2009); *Vibrio cholerae* (PDB:3QMN; Halavaty et al. 2012), and the NCS ligands function in WinCoot. CoA molecules were modelled to fit the electron density in WinCoot, and the occupancies of the molecules refined using Phenix Refine. In total, three CoA molecules were observed in the asymmetric unit. Potassium ions within the molecule were placed in a similar manner, and co-ordination, valance, and bond distances were confirmed using the “check my metal server” (as described in section 2.2.18). The final model was refined to an R-value of 0.185 and R-free of 0.224, with full data collection statistics listed below in Table 3.1.
Table 3.1 - *NmAcpS* diffraction data and model statistics.

<table>
<thead>
<tr>
<th></th>
<th>Apo-AcpS</th>
<th>AcpS:CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>48.06-2.00 (2.05-2.00)</td>
<td>46.34-1.75 (1.78-1.75)</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
<td>a = 48.06, b = 90.07, c = 93.05</td>
<td>a = 47.51, b = 92.69, c = 92.00</td>
</tr>
<tr>
<td>Total observations</td>
<td>200760 (14890)</td>
<td>125717 (7092)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>28036 (2120)</td>
<td>41271 (2326)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.2 (7.0)</td>
<td>3.0 (3.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100.0 (99.9)</td>
<td>98.2 (98.8)</td>
</tr>
<tr>
<td>Mean I/sigma (I)</td>
<td>9.1 (3.4)</td>
<td>9.8 (2.3)</td>
</tr>
<tr>
<td>CC (1/2)</td>
<td>0.995 (0.970)</td>
<td>0.995 (0.895)</td>
</tr>
<tr>
<td>R-pim</td>
<td>0.046 (0.122)</td>
<td>0.38 (0.220)</td>
</tr>
<tr>
<td>R-meas</td>
<td>0.123 (0.325)</td>
<td>0.070 (0.401)</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.144 (0.301)</td>
<td>0.059 (0.331)</td>
</tr>
<tr>
<td>R-work</td>
<td>0.185</td>
<td>0.216</td>
</tr>
<tr>
<td>R-free</td>
<td>0.224</td>
<td>0.250</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>3221</td>
<td>3408</td>
</tr>
<tr>
<td>RMSD bonds (Å)</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>RMSD angles (°)</td>
<td>0.980</td>
<td>0.966</td>
</tr>
<tr>
<td>Ramachandran favoured (%)</td>
<td>97.6</td>
<td>97.8</td>
</tr>
<tr>
<td>Ramachandran allowed (%)</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.

3.2.2 Overall structure of *NmAcpS* complexes

The crystal structures of apo *NmAcpS* and the *NmAcpS*:CoA complex contain an α/β protein arranged as a homo-trimer, a quaternary structure that is consistent with other bacterial AcpSs (Dym et al. 2009, Dall'aglio et al. 2011, Halavaty et al. 2012). Each monomer in the asymmetric unit consists of 125 residues, comprising the full amino acid sequence of *NmAcpS*. The structure of *NmAcpS* is similar to that of other bacterial
AcpSs, with each monomer consisting of 5 β-strands and 5 α-helices (Figure 3.3). The 5 β-strands of each monomer are arranged to form two antiparallel β-sheets, which flank helix α4. The first β-sheet formed by strands β1, β5, and β4 (sheet A), and the second formed by strands β2 and β3 (sheet B), pack diagonally against α4, similar to a hotdog fold. Helices α1, α2, α3, α4, and a small helical turn form an antiparallel four helix bundle. Helix α5 lies outside this bundle on the outer edge of the monomer, connecting the two antiparallel β-sheets. Helices α1, α2, and α3 in two of the three NmAcpS monomers have high B-factors in comparison to the rest of the model, while high B-factors are observed in helix α4 in only one chain. Similar disorder has been reported in other bacterial AcpSs (Dym et al. 2009, Dall'aglio et al. 2011, Halavaty et al. 2012).

The average B-factors of CoA bound NmAcpS is roughly 10% lower than that of the apo structure, yet not all those helices displaying average B-factors are supported by crystal contacts, which suggests NmAcpS is somewhat flexible in the absence of bound CoA and ACP.

The three NmAcpS monomers of the asymmetric unit are related by 3-fold non-crystallographic pseudo-symmetry. The three-stranded β-sheet (sheet A) of each NmAcpS monomer lies at the centre of the trimeric complex and serves as the site of two identical protein-protein interfaces that lie opposite one another (Figure 3.3). The first interface occurs through strand β1, which aligns parallel to strand β4 of the monomer, flanking one side of sheet A. The second interface is formed through strand β4, which aligns parallel to strand β1 of the adjacent monomer, flanking the opposite side of sheet A.
The arrangement of the three monomers forms a long hydrophobic barrel that runs the length of the complex (Figure 3.3). Formation of the trimeric complex is largely stabilised by hydrogen bonding between the β strands of sheet A, however Glu124 of
strand β5 also forms a hydrogen bond with Ile2 of β1 on the opposing monomer. Hydrophobic packing and possible aromatic interactions between residues Phe120 on strand β5 of each monomer may also aid in stabilising the trimer, however Phe120 does not appear to be conserved in other bacterial AcpS enzymes, so it is unlikely to play a significant role in the enzyme’s quaternary structure.

Gel filtration chromatography indicates the biological assembly of *Nm*AcpS is that of a trimer, which is consistent with other bacterial AcpSs and other type II PPTases (Lambalot et al. 1996, Dym et al. 2009, Dall'aglio et al. 2011, Halavaty et al. 2012), with active sites formed through the dimerisation of two monomers. The active site and CoA binding pocket lies in a depression adjacent to the interface between the three-stranded β-sheets of each monomer, with half of the active site formed by each monomer, and formation of a trimer creating three active sites.

### 3.2.3 *Nm*AcpS Co-enzyme A binding site and interactions

The crystal structure of *Nm*AcpS bound to CoA reveals that the adenine moiety and the 3’ phosphate of the CoA molecule lie in a pocket formed by the intersection of the 3-stranded β-sheets of two monomers (Figure 3.4), the loop connecting helix α4 and strand β1 of one molecule, and the hair pin loop connecting strands β1 and β2 of the opposing monomer. The 6’ amine group of the adenine ring appears to form hydrogen bonds with residues Leu83 and Gly63, while the 3’ phosphate is stabilised by His80 and Lys51, and the 2’ hydroxyl of the adenine adjacent ribose is within hydrogen bonding distance of Pro86 and Met110. The most numerous interactions appear to occur between the oxygen atoms of the pyrophosphate of the CoA molecule, with residues Asp8, Glu57, Lys61 and Ser111 positioned to form hydrogen bonds with this moiety. The position of the remaining phosphopantetheine arm appears to be dictated by
hydrophobic interactions, with one possible hydrogen bond formed between Ile67 and oxygen atom OAP of the CoA molecule.

Electron density for varying parts of the phosphopantetheine arm after the diphosphate moiety is disordered in the NmAcpS:CoA complex. However, the terminal thiol atom of the CoA molecules appear pointed towards Arg29, positioned between three phenylalanine residues in a conformation similar to that observed in the structure of the CoA bound Bacillus subtilis AcpS (BsAcpS; PDB:1F7L). Interestingly, the conformations of the CoA molecule in both the NmAcpS and BsAcpS bound complexes are different to that observed in Streptomyces coelicolor AcpS (ScAcpS; PDB:2WDS). Superposition of the ScAcpS:CoA complex reveals the conformation of CoA present in this structure is incompatible with NmAcpS and BsAcpS, causing a clash with residue Ile67 (Ile68 in BsAcpS). This indicates differences in the substrate binding pockets may exist between some bacterial AcpS enzymes, thus future drug design efforts should take into account such variation.

The NmAcpS:CoA complex contains two highly coordinated water molecules between the diphosphate groups of two of the three CoA molecules in the model, positioned between Asp8, Glu57, and the two phosphates of the diphosphate moiety. Whilst water molecules are present in two of the three active sites, placement of these molecules does not appear to alter the CoA conformation observed within the active sites. The role of these water molecules is unknown, however divalent cations are present in the same location in the BsAcpS:CoA and ScAcpS:CoA structures, and appear essential for AcpS catalytic activity, with the preferred co-factor of E. coli AcpS being Mg$^{2+}$, yet E. coli AcpS is able to utilise Mn$^{2+}$ in the absence of Mg$^{2+}$ ions, albeit at a significantly reduced catalytic rate (Elovson & Vagelos, 1968).
3.2.4 Putative Aeryl Carrier Protein binding sites and interactions with NmAcpS

To further characterise the active site of NmAcpS, we attempted to co-crystallise recombinant NmAcpS with apo-ACP. However, crystals of this complex had not been obtained at the time of writing, and no alternate structural information regarding this complex is available, thus the exact interactions between NmAcpS and N. meningitidis ACP remain unknown. However, superposition of the crystal structures of AcpS from B. subtilis (PDB:1F80) and S. aureus (PDB:4DXE) in complex with ACP and NmAcpS, indicate that these proteins bind through the formation of a number of salt bridges. Residues Arg15, Lys21, Arg29, and Lys45 of NmAcpS are conserved amongst most Gram negative and Gram positive bacteria, with the exception of the Corynebacterineae family of bacteria (including Mycobacterium tuberculosis), and appear to form salt bridges with conserved acidic residues of ACP (see Appendix 1 for sequence alignments).

The NmAcpS:ACP model generated through superposition of these structures shows that Arg14 from one of the monomers forming half of the NmAcpS active site is positioned to form salt bridges with Asp36 and Asp39 of N. meningitidis ACP (NmACP). Within the same NmAcpS monomer, Lys21 also appears to form a salt bridge with Glu42 of NmAcp, while Arg29 is positioned to form salt bridges with both Glu48 and Glu49 of NmAcp. A single salt bridge may also be formed through Lys45 of the opposing NmAcpS monomer, and residue Asp31 of NmAcp. Parris et al. (2000) suggest that the interactions between Arg14 of Bacillus subtilis AcpS and Asp36 and Asp39 of Bacillus subtilis ACP are pivotal in ensuring Ser37 of ACP, the residue to which the P-pant moiety of CoA is attached, is correctly positioned within the AcpS active site.
Figure 3.4 - The active site of *N. meningitidis* AcpS in complex with CoA. (A) CoA is bound to *Nm*AcpS at the interface between two *Nm*AcpS monomers (differing monomers are coloured green and silver). The adenine ring of the CoA molecule appears to bind Leu83 and Gly64, while the 3' phosphate is stabilised by His80 and Lys51. The most numerous interactions appear to occur with the pyrophosphate of the CoA molecule, with residues Asp8, Glu57, Lys61 and Ser111 positioned to form hydrogen bonds with this moiety. The position of the remaining phosphopantetheine arm appears to be dictated by hydrophobic interactions, with a single hydrogen bond formed between Ile67 and oxygen atom OAP of the CoA molecule. (B) A 2D representation of the interactions between *Nm*AcpS and CoA. CoA is coloured purple; the coordinated water molecule is represented by a cyan coloured sphere; hydrogen bonds are represented by green dashed lines; hydrophobic contacts are shown as semi-circular arcs.
Despite a large degree of structural similarity between \( NmAcpS \) and AcpS of other bacterial species (Figure 3.5), their sequence identity is surprisingly low, ranging from 28 to 42\% (Appendix 1). The most highly conserved region is that of helix \( \alpha_4 \), which forms a portion of the active site, and residues which are indicated to interact with ACP based on superposition of the ACP bound \( B. \ subtilis \) (PDB:1F80) and \( S. \ aureus \) (PDB:4DXE) AcpS structures. The conserved nature of these residues implies they have a critical role, yet Arg14, Lys21, and Arg29 appear to be replaced with acidic residues in \( M. \ tuberculosis \) and are thus unable to form the same interactions. This casts some doubt on whether the interactions observed between Arg14, Lys21, Arg29, and ACP in the \( B. \ subtilis \) and \( S. \ aureus \) AcpS:ACP crystal structures represent those formed \textit{in vivo}.

\textbf{Figure 3.5 - The structure of AcpS is highly conserved amongst bacterial homologues.} Despite sequence identities ranging from \( \sim35 \) to 60\% compared to that of \( NmAcpS \), the secondary structure of bacterial AcpS enzymes is well conserved, as shown by superposition of a monomer of \( NmAcpS \) (cyan), \( M. \ tuberculosis \) (PDB:3NE1, red), \( S. \ aureus \) (PDB:4DXE, yellow), \( S. \ coelicolor \) (PDB:2JCA, orange), \( S. \ pneumoniae \) (PDB:1FTE, green), and \( V. \ cholerae \) (PDB:3QMN, pink) AcpS structures at 0\° (A) and 90\° (B) rotation.
Mutation of Arg14 to alanine (NmAcpS numbering) and the reciprocal ACP residues of *Streptomyces coelicolor* ACP and AcpS show Arg14 is not essential for binding CoA or apo-ACP, with little change in the respective dissociation constants compared to wild type *Streptomyces coelicolor* AcpS (Dall'aglio et al. 2011). Yet despite its limited effect on CoA or ACP binding, mutation of this residue to alanine, and thus abolishing the salt bridges formed either side of Ser37, reduces AcpS catalytic activity to approximately 15% of that of the wild type enzyme, suggesting Arg14 is indeed important for correctly positioning Ser37 into the active site of AcpS (Parris et al. 2000, Dall'aglio et al. 2011). The ACP/AcpS binding studies performed by Dall'aglio et al. (2011) utilising *Streptomyces coelicolor* mutants did not directly examine the role of Lys21 from AcpS in interactions with ACP. However, mutation of Asp39, which appears to interact with Lys21, to alanine resulted in a 9-fold reduction in AcpS binding and completely abolished the conversion of apo-ACP to holo-ACP by AcpS. These studies confirm that both Arg14 and Lys21 play a critical role in the formation of an AcpS:ACP complex, and that *M. tuberculosis* AcpS binds apo-ACP via different residues than those of most other bacteria. Although no mutagenesis studies have examined the roles of Arg29 and Lys45, the crystal structures of AcpS bound to ACP clearly indicate that they play a role in AcpS:ACP complex formation (Parris et al. 2000). This notion is reinforced by the conserved nature of these residues, and as the *M. tuberculosis* AcpS/ACP interaction appears atypical, the lack of these residues in *M. tuberculosis* and other members of the Corynebacterineae family is not indicative of a lack of function.

### 3.2.5 Putative NmAcpS reaction mechanism and active site

Following binding of CoA and Mg\(^{2+}\) ions, the catalytic transfer of the 4’-phosphopantetheine onto Ser37 (NmACP numbering) is thought to occur by a three step mechanism (Figure 3.6). Firstly, the receptive serine of ACP is deprotonated. The deprotonated...
serine then initiates a nucleophilic attack on the β-phosphate of the bound CoA, transferring the P-pant group to Ser37. Finally, the α-phosphate of the resulting 3',5'-ADP molecule is protonated and holo-ACP dissociates from AcpS. Parris et al. (2000) suggest that the initial deprotonation of Ser37 from ACP occurs via an activated water molecule, which itself is deprotonated by either another water molecule or the ACP residue Asp36. The activated serine then attacks the β-phosphate of CoA, cleaving the phosphodiester bond and transferring the P-pant moiety onto ACP. The negative charge of the newly formed 3',5'-ADP molecule is stabilized by Lys62, His105, and either one or both of the bound Mg$^{2+}$ ions, and Lys62 or a solvent molecule donates a proton to the 3',5'-ADP α-phosphate. Based on analogy with human ACP synthase and the B. subtilis PPTase Sfp, Dall'aglio et al. (2011) suggest that Glu57, aided by a helix-dipole effect from the α2 helix on ACP and proximity to the cationic Mg$^{2+}$, abstracts the proton from Ser37 instead of an activated water molecule. Glu57 does appear to be both highly conserved and essential for catalytic activity, with mutation of this residue to an alanine in ScAcpS completely abolishing the phosphopantetheinylation of apo-ACP. Based on analogy with human ACP synthase, it also appears likely that Lys62 is required for protonation of the 3',5'-ADP α-phosphate following pyrophosphate cleavage, not a solvent molecule, with mutation of the homologous lysine residue of human ACP synthase to alanine resulting in a 3-fold reduction in catalytic activity, but little reduction in affinity for CoA or Mg$^{2+}$ ions, which suggests the primary role of Lys62 is protonation of the α-phosphate (Bunkoczi et al. 2007). Regardless of the exact mechanism by which Ser37 is activated, the three step reaction and residues involved appear to strongly mimic active site architecture and transfer mechanism of the human AcpS, a group II PPTase (Figure 3.6).
Figure 3.6 - The proposed catalytic mechanism of AcpS. (A) Parris et al. (2000) suggest Ser37 of ACP is deprotonated via an activated water molecule. The activated serine then attacks the β-phosphate of CoA, cleaving the phosphodiester bond and transferring the P-pant moiety onto ACP. The negative charge of the 3',5'-ADP molecule formed is stabilized by Lys62, the main chain amide of Asp112, and a bound Mg\(^{2+}\) ion, and Lys62 or a solvent molecule donate a proton to the 3',5'-ADP α-phosphate. Based on analogy with human ACP synthase and the B. subtilis PPTase Sfp, Dall'aglio et al. (2011) believe that Ser37 of ACP is deprotonated by Glu57, aided by a helix-dipole effect from the α2 helix on ACP and proximity to the cationic Mg\(^{2+}\), instead of an activated water molecule. (B) Superposition of NmAcpS (monomers coloured silver, cyan, and magenta) and human AcpS (PDB:2C43, red), a group II PPTase, reveals a similar active site and CoA binding site. Note: Figure (A) adapted from Parris et al. (2000).

3.2.6 AcpS inhibitors

Existing inhibitors of AcpS are limited to the inhibitor Sch538415, a series of anthranilic acid derivatives, and carbonthioamide based compounds. Sch538415, an anthraquinone compound, has been demonstrated to inhibit S. aureus AcpS with an IC\(_{50}\) value of 4.19 µM, and possess some degree of antimicrobial activity as demonstrated via disc assay. No minimum inhibitory concentration or structural data relating to the possible binding mechanism of Sch538415 have been reported. As such, the antimicrobial potential of Sch538415, and whether its reported antimicrobial activity is
due to inhibition of AcpS alone or other biological molecules, remains unknown. However, with further characterisation of the antimicrobial activity of Sch538415, and determining the site at which it binds AcpS, Sch538415 could be a useful lead compound in the development of future AcpS inhibitors, as it not only demonstrates some degree of existing antimicrobial activity, but the tricyclic backbone theoretically allows the modification of the molecule through attachment of various functional groups (Chu et al. 2003).

The anthranilic acid derivatives identified to inhibit Bacillus subtilis AcpS by Gilbert et al. (2004) and Joseph-McCarthy et al. (2005) have been studied in far greater detail than Sch538415. X-ray crystallography of B. subtilis AcpS in complex with four selected anthranilic acid derivatives shows that these compounds are competitive inhibitors that occupy the same binding site as the adenine, ribose, and 3’-phosphate moieties of CoA at the dimer interface. These inhibitors appear to be largely stabilised by van der Waals interactions between Lys61 of one monomer and Lys85 and Pro86 of the opposing monomer (NmAcpS numbering). The anthranilic compounds are within hydrogen bond distance of residues Ser109 and Met110 (Ser102 and Ile103 in B. subtilis), which also appear to form hydrogen bonds with the adenine moiety and adenine adjacent ribose of the CoA bound AcpS structures.

Some of the anthranilic compounds that have been developed exhibit a very high affinity for B. subtilis AcpS, with IC$_{50}$ values of the most potent compounds ranging from 0.13 to 0.9 µM. Minimum inhibitory concentrations have not been reported for all of these compounds, however of those molecules for which it has been reported, MIC values range from 25 to ≥ 200 µM. The large variation in reported values shows that the
MIC value is not directly correlated to the IC$_{50}$ value, which suggests these inhibitors have multiple targets.

One issue not addressed in these studies is the role of alternate PPTases such as Sfp of *B. subtilis*. Sfp has been demonstrated to catalyse the phosphopantetheinylation of apo-ACP in the absence of AcpS, and could account for the relatively weak antimicrobial activity exhibited by these compounds. To combat this limited antimicrobial activity Foley et al. (2014) developed 2-pyridinyl-N-(4-aryl)piperazine-1-carbothioamide based compounds that simultaneously target *B. subtilis* Sfp and AcpS. The most promising of these compounds, 4-(3-Chloro-5-(trifluoromethyl)pyridin-2-yl)-N-(4-methoxypyridin-2-yl)piperazine-1-carbothioamide trifluoroacetate (ML267), displayed MIC$_{50}$ values ranging from 1.2 to 4 µM against methicillin resistant *S. aureus* strains, as well as low affinity for human PPTase (>114 µM). However, ML267 has been shown to be rapidly expelled by the AcrAB–TolC efflux pump in *E. coli*, and while antimicrobial activity can be partially restored in AcrAB–TolC efflux defective mutants (12 µM), it is still at least three fold less potent against wild type *E. coli*.

Fortunately, the issues of alternate phosphopantetheinyl transferases and antimicrobial export through antimicrobial efflux that plague Sch538415, anthranilic inhibitors, and ML267, may not apply to *N. gonorrhoeae* and *N. meningitidis*, as neither appear to possess PPTases other than AcpS (Donadio et al. 2007), thus inhibition of this enzyme should prevent activation of apo-ACP. Additionally, efflux of antimicrobials does not appear to occur via the AcrAB–TolC efflux pump in *N. gonorrhoeae* or *N. meningitidis* as it does in *E. coli*, which may increase the antimicrobial potential of these molecules compared to wild type *E. coli*. However, *N. gonorrhoeae* and *N. meningitidis* possess at least four other efflux systems, FarA-FarB, MtrC-MtrD-MtrE, MacA-MacB, and NorM.
which have all previously displayed the ability to export antimicrobials (Rouquette-Loughlin et al. 2003, Kamal et al. 2007, Kamal et al. 2010).

Whether the aforementioned compounds are exported by these efflux pumps is yet to be determined. Regardless, Sch538415, the anthranilic inhibitors, and to a greater extent ML267, all demonstrate that despite possessing a catalytic mechanism and active site architecture similar to the human orthologue, AcpS is a valid drug target, and the possibility does exist to develop inhibitors that exhibit both low affinity for human AcpS and potent antimicrobial activity. The X-ray crystallographic structures presented here may aid in that discovery.

3.3 Neisseria FabD

3.3.1 Structure determination

As per AcpS, the gene encoding FabD from Neisseria meningitidis strain FAM18 (NmFabD; No. YP_974419.1) was amplified from genomic DNA (ATCC catalogue number: 700532D-5) and cloned into the expression vector pMCSG21 by LIC (section 2.2.5). Colonies containing the recombinant plasmid were confirmed by spectinomycin resistance and colony PCR. Recombinant plasmid was then isolated from the positive clones and confirmed to be free of mutation by DNA sequencing. E. coli BL21(DE3) pLysS cells were transformed with recombinant plasmid encoding NmFabD. Recombinant NmFabD was solubly over-expressed using auto-induction media (section 2.2.12), purified by HisTrap HP Ni⁺ affinity chromatography and S200 26/60 size exclusion chromatography (section 2.2.13), and concentrated to ~35 mg/mL via Amicon centrifugal device. Purified NmFabD was assessed to be ~90% homogeneity by SDS-PAGE (section 2.2.15) (Figure 3.7).
Preliminary crystal screening was performed via the hanging drop vapour diffusion technique utilising commercially available crystallization screens (section 2.2.17). Clusters of small needle shaped crystals were observed in ammonium sulfate or lithium sulfate conditions lacking PEG and buffered to a pH range of 7.5 to 8.5 (Crystal screen condition no. 4, Crystal screen II condition no. 32, Proplex II conditions no. 27, 28, and 39) after approximately one month, yet were judged too small for collection of X-ray diffraction data. Crystal optimisation based around these conditions using a pH range of 5.5 to 9.5 and with or without 10% glycerol, resulted in no obvious improvements in morphology. As crystals appeared to favour sulfate-containing solutions, NmFabD was screened against cesium sulfate, magnesium sulfate, potassium sulfate, and sodium sulfate solutions buffered to pH 7.0, 8.0, and 9.0 with 100 mM TRIS. Slightly larger needle shaped crystals were observed in 1.5 to 2 M sodium sulfate at pH 7.0, 8.0, and 9.0, however these crystal were still too small for data collection.

To increase the number of potential nucleation sites and thus induce the formation of larger single crystals rather than clusters, microseeding was employed. Briefly, crystals were harvested by pipette and diluted in reservoir solution. The collected crystals were then pulverised into microseeds by vortex mixer and a single freeze/thaw cycle. When conducting optimisations, 1.0 μL of this seed stock was added to 80 μL of concentrated protein, before 1.5 μL protein solution containing microseeds was combined with 1.5 μL reservoir solution on a circular cover slip and suspended over 300 μL reservoir solution (section 2.2.17). Serial seeding (utilising subsequent crystals as microseeds) using these conditions resulted in the formation of diffraction quality crystals in 1700 mM sodium sulfate and 100 mM TRIS at pH 8.0 following three rounds of microseeding.
Figure 3.7 - Expression and purification of *N. meningitidis* FabD (NmFabD). NmFabD was purified by affinity chromatography (A) followed by size exclusion chromatography (B). Pink line indicates the point of sample injection; blue line represents absorption in mAU; green line indicates the concentration of elution buffer (His buffer B). Protein expression and purification were assessed by SDS-PAGE (C), with samples loaded as follows: protein standards (i), whole cell extract (ii), cell lysate (iii), affinity column flowthrough (iv), affinity column elution (v), cleavage of affinity tag by TEV protease (vi), S200 fractions (vii, viii, ix), and concentrated protein (x). A crystal of NmFabD mounted in cyroprotectant (D) and collected diffraction data at 1° (E) and 90° (F) rotation.
Prior to data collection, the *Nm* FabD crystal was cryoprotected in a solution consisting of 1700 mM sodium sulphate, 100 mM TRIS at pH 8.0, and 25% glycerol, and flash cooled in liquid nitrogen. X-ray diffraction data were collected at the Australian synchrotron on the MX-2 beamline. A total of 180° of X-ray diffraction data were collected at 0.5° oscillations and a wavelength of 0.951 Å. Diffraction data were indexed and integrated in iMosflm, and scaled using Aimless to a resolution of 2.70 Å. The space group of the crystal was ambiguous, displaying either $P2_12_12_1$ symmetry with the unit cell parameters $a = 36.62$, $b = 93.07$, $c = 171.04$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 90^\circ$, or $P2_1$ symmetry with the unit cell parameters $a = 36.62$, $b = 172.73$, $c = 93.03$ Å, $\alpha = 90$, $\beta = 89.37$, $\gamma = 90^\circ$ displaying pseudo-merohedral twinning with the twin operator $h$, $-k$, $-l$. Twinning was suspected due to split reflections observed in the high resolution limits of the diffraction pattern (Figure 3.7).

To ensure the correct space group, diffraction data were integrated and scaled in both $P2$ and $P222$ point groups, in addition to molecular replacement using Phaser and a monomer of FabD from *Burkholderia pseudomallei* (PDB:3EZO; Baugh et al. 2013) as a search model being attempted in all possible space groups. The only solution found using the data integrated in the point group $P222$ was $P2_12_12_1$. The $P2_12_12_1$ solution contained two molecules in the asymmetric unit based on a molecular weight of 32 kDa, with a solvent content of 47.27% and a Matthews coefficient of 2.33 Å$^3$/Da. Model building and refinement (section 2.2.18) failed to reduce the R and R-free values by a significant amount (R and R-free $\sim$0.45), confirming $P2_12_12_1$ was the incorrect space group. All subsequent model building and analysis was conducted using data integrated and scaled as space group $P2_1$. Using a molecular weight of 32 kDa, four *Nm* FabD molecules were expected in the asymmetric unit of the data integrated and scaled as $P2_1$ symmetry, giving a Matthews coefficient of 2.31 Å$^3$/Da and a solvent content of
46.82%. Following model building and structure refinement (section 2.2.18), 307 of 308 residues were placed, with terminal histidine residues not fully defined in the electron density map, and the final R and R-free values were found to be 0.272 and 0.285 respectively (see Table 3.2 for crystallographic and data collection statistics). Attempts to obtain a structure of NmFabD in complex with CoA by either co-crystallisation or soaking failed to yield diffraction quality crystals using the same conditions and screens as apo NmFabD.

### 3.3.2 Overall structure of NmFabD

The crystal structure of NmFabD contains four molecules of 307 amino acids within the asymmetric unit. The NmFabD monomer is comprised of 12 α-helices and 9 β-stands, arranged into two subdomains containing distinct folds that flank the putative active site of the enzyme. The smaller of the two subdomains (residues 123-203) mimics a ferredoxin fold observed in acylphosphatases (Katz et al. 1999, Keatinge-Clay et al. 2003), and is comprised of a four-stranded anti-parallel β-sheet capped by α-helices α6 and α7. The second and larger of the two domains is non-contiguous (residues 1-123 and 203-307), and comprised of five β-strands arranged to form a central parallel β-sheet surrounded by 10 α-helices and capped by a β-hairpin (Figure 3.8). The two domains are joined via short loops connecting helices α6 and α9 of the larger subdomain to strands β3 and β6 of the smaller domain respectively. The tertiary and secondary structures of FabD enzymes are well conserved (Figure 3.9), with an RMSD of 1.06 to 1.90 Å over ~300 amino acids, despite sequence identities ranging from ~35 to 60% compared to that of NmFabD (Appendix 1). Although the sequence of these enzymes can vary greatly, there are at least two conserved sequence motifs, 8PGQGXQ13 and 88GHSLGE93 (NmFabD numbering), that appear important for substrate specificity, catalytic activity, or both.
### Table 3.2 - *Nm*FabD diffraction data and model statistics.

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<tr>
<td>Unit cell angle (°)</td>
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<tr>
<td>Ramachandran outliers (%)</td>
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</tbody>
</table>

Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.
Figure 3.8 - Tertiary and secondary structure elements of *N. meningitidis* FabD.

*NmFabD* is a monomeric protein containing 12 α-helices and 9 β-strands. The secondary structure elements of *NmFabD* at 1° (A), 90° (B), and 180° (C) rotation. A 2D schematic of the secondary structure topology (D) and protein sequence of *NmFabD* (E). α-helices, β-strands, and loops are coloured cyan, red, and magenta, respectively.
3.3.3 Putative active site architecture and catalytic mechanism of *NmFabD*

Efforts to obtain *NmFabD* in complex with CoA failed to yield crystals, preventing confirmation of the active site of *NmFabD* by X-ray crystallography. However, the active site and catalytic mechanism of FabD enzymes appears well conserved amongst bacteria (Serre et al. 1995, Keatinge-Clay et al. 2003, Ghadbane et al. 2007, Li et al. 2007, Zhang et al. 2007, Natarajan et al. 2012), and other FabD enzymes in complex with CoA can be superimposed onto the structure of apo-*NmFabD*, thus the active site and important catalytic residues can be somewhat conferred by homology (Figure 3.10).

The formation of malonyl-ACP catalysed by FabD is thought to occur via a ping-pong type reaction mechanism, in which a FabD:malonate complex is first formed, followed by...
by transfer of the malonyl group from the active site serine of FabD onto the P-pant arm of holo-ACP (Figure 3.10).

Figure 3.10 - The active site of *N. meningitidis* FabD, and putative reaction mechanism. (A) The active site of *Nm*FabD is formed predominantly by residues Gln10, Ser90, Leu91, Arg115 and His199. (B) Superposition of CoA and malonic acid (yellow) from *E. coli* FabD (PDB:2G2Z) indicates Gln164, Lys187, and Arg285 residues shift to accommodate the adenine ring of the CoA, which forms a number of bonds with surrounding residues (yellow dashes). (C) The reaction mechanism suggested by Oefner et al. (2006). FabD is thought to catalyse the formation of malonyl-ACP via a ping-pong reaction mechanism, in which a FabD:malonate complex is first formed, followed by transfer of the malonyl group from the active site serine of FabD onto the P-pant arm of holo-ACP. Figure (C) adapted from Oefner et al. (2006).
The active site of FabD enzymes lies at the base of a cavity between the two subdomains of the enzyme, and is lined by the catalytically important residues Gln10, Ser90, Arg115, and His199 (NmFabD numbering). Upon binding CoA, residues Gln10 and Leu91 are thought to shift closer together, activating an oxyanion hole. The malonyl group of malonyl-CoA enters the oxyanion hole, where the substrate is stabilised by hydrogen bonding between the carbonyl oxygen of the thioester and the main chain amide of Gln11, and a salt bridge between Arg115 and the carboxylate of the malonyl group. The binding of CoA triggers the extraction of a proton from Ser90 by His199, activating the nucleophile. Ser90 then initiates nucleophilic attack on the carbonyl of the malonyl group, attaching the thioester to FabD and forming a tetrahedral intermediate. The resulting charge appears to be supported by the oxyanion hole formed by Gln10 and Leu91, and a positive dipole moment due to proximity to helix α5. His199 subsequently donates a proton to the bound CoA molecule, causing CoA to dissociate from FabD, forming the FabD:malonate complex, and completing the first part of the ping-pong reaction (Oefner et al. 2006, Misra et al. 2009).

The hydroxyl oxygen atom (OG) of Ser90 of NmFabD is positioned ~3.3 Å from one of the Arg115 side chain nitrogen atoms (NH2), and ~2.3 Å from a His199 side chain nitrogen (NE2). These residues appear to form H-bonds, which act to support deprotonation of the active site serine and its subsequent nucleophilic attack. The position of Ser90 in the \textsuperscript{88}GHSLGE\textsuperscript{93} motif, a highly conserved nucleophilic elbow, between strand β2 and helix α5 presumably allows the residue some degree of rotation, while the dipole of the nucleophilic elbow at the N-terminal of helix α5 assists in stabilising the oxyanion hole.
In the second part of the reaction, holo-ACP binds the FabD:malonyl complex. Thus far, no X-ray crystallography or NMR structures of FabD bound to ACP have been published, however docking studies with FabD and apo-ACP from *Helicobacter pylori* (HpFabD) by Zhang et al. (2007) suggest that the binding of ACP to FabD pushes Gln10 towards Leu91, reforming the oxyanion hole. Following either dissociation of CoA or ACP binding, the active site serine bound to the malonyl group rotates to accommodate the P-pant moiety of holo-ACP, which as Zhang et al. (2007) suggest, binds to FabD in a similar way to malonyl-CoA, with the terminal thiol positioned in the newly reformed oxyanion hole. Once bound to FabD, the terminal thiol of the holo-ACP P-pant arm protonates His199 of FabD, becoming an active nucleophile. The active thiol then attacks the ester linking Ser90 and the malonyl group, inducing a tetrahedral transition state, which is supported by the oxyanion hole. Finally, the active site serine of FabD is protonated by His199 and the tetrahedral intermediate collapses, releasing malonyl-ACP (Keatinge-Clay et al. 2003, Oefner et al. 2006, Misra et al. 2009) (Figure 3.10).

### 3.3.4 Potential *NmFabD* ACP/CoA binding interactions

The interactions between FabD and its substrates malonyl-CoA and ACP are yet to be fully characterised. The X-ray crystallographic structure of *EcFabD* in complex with CoA and malonate indicates that the adenine ring of CoA binds between strand β5 of the smaller subdomain and helix α12 of the larger subdomain, stretches along the cleft between the two subdomains towards the active site, and forms hydrogen bonds with a number of conserved residues (Oefner et al. 2006). Superposition of the *EcFabD*:CoA structure and that of *NmFabD*, shows the N6A nitrogen atom of the CoA adenine ring interacts with the hydroxyl groups of Ser161 and Pro162 (*NmFabD* numbering), with additional bonds are formed with residues Arg188 and Arg285. An oxygen atom of the
CoA 3’ phosphate engages in hydrogen bonding with residue Arg188, and the oxygen atom between the adenine ribose and the pyrophosphate is positioned to form a hydrogen bond with the terminal NH2 nitrogen atom of Arg285 (~2.8 Å apart). The remainder of the P-pant moiety is stabilised by hydrogen bonds between Gln164 and the OAP and N8P atoms of CoA, the side chain nitrogen of Asp158, and O5P of the CoA molecule (Figure 3.10). Superposition of NmFabD and the EcFabD:CoA:malonate structure also suggests that the Gln166, Lys187, and Arg285 residues shift to accommodate the adenine ring of the CoA, thus CoA binding induces some degree of conformational change.

Docking of apo-ACP to FabD by Zhang et al. (2007) suggests that the electropositive residues Lys181, Arg187, Lys278, and Lys282 of HpFabD bind ACP, with the ACP serine residue that binds the P-pant arm positioned near the entrance to the active site of FabD. In the model suggested by Zhang et al. (2007), the ACP residues Asp35 and Leu37, that are located either side of the catalytic Ser36, bind to Gly11 and Val276 of HpFabD adjacent to the oxyanion hole, positioning the ACP catalytic serine close to the active site of FabD. In NmFabD Lys278 is substituted with alanine (Ala280), however Lys277 of NmFabD lies ~5 Å from Lys278 of HpFabD, and may fill the same role. Docking studies performed by Keatinge-Clay et al. (2003) and Natarajan et al. (2012) suggest that ACP binds at a similar location yet is rotated approximately 180°, with the receptive ACP serine positioned ~20 Å from the FabD active site, near the apparent binding site of the CoA adenine ring, with the ACP residues equivalent to Asp35 and Leu37 interacting with S. coelicolor FabD residues Lys298 and Thr299. This conformation appears to be in agreement with the interactions observed in the EcFabD:CoA:malonate complex, with the pyrophosphate of the CoA molecule
(beginning of the 4′-phosphopantetheine arm) also positioned ~20 Å from the FabD active site, however neither model is supported by experimental data.

The structure of EcFabD in complex with CoA and the malonate ion fails to provide an adequate picture of the interactions between FabD and malonyl-CoA as the malonyl group has been cleaved from the CoA molecule, thus the structure more closely represents an intermediate state between the two stages of the ping-pong reaction mechanism. Likewise, the interactions between FabD and ACP remain largely uncertain, as the docking studies conducted with FabD and ACP reported differing orientations of ACP, and thus far, mutational analysis has not clarified these issues. Therefore any additional studies, particularly structural models that demonstrate malonyl-CoA or ACP binding interactions, would be incredibly useful in clarifying these issues.

3.3.5 FabD inhibitors

As mentioned earlier, few inhibitors of FabD have been reported, and the antimicrobial properties and binding interactions of the majority of these compounds have been poorly characterised. Shen et al. (2010) identified, several phenylbenzoic acid based molecules they suggest strongly inhibit EcFabD. Based on molecular docking studies, these molecules appear to bind near the active site of FabD, interacting with Arg115 (NmFabD numbering) and Gln10, in a manner which could potentially disrupt substrate binding and/or the oxyanion hole. However, the antimicrobial and inhibitory properties of these compounds is still unknown as no IC₅₀ or MIC values have been reported for any of the inhibitors identified, and based on photographs of the antimicrobial disc diffusion assays, their antimicrobial activity appears far weaker than the ampicillin control (Shen et al. 2010).
The aporphine alkaloid FabD inhibitor corytuberine is perhaps the most characterised of all reported FabD inhibitors. Identified by Liu et al. (2006) in a high throughput screening assay, corytuberine was found to inhibit FabD of *H. pylori* with an IC$_{50}$ value of approximately 33 µM, yet like the inhibitors reported by Shen et al. (2010), no accompanying MIC values have been provided. The potential binding site of corytuberine and the related aporphine alkaloid compounds boldine, dicentrine, and glaucine were investigated by Rohini et al. (2012). Their molecular docking simulation suggested that corytuberine, boldine, dicentrine, and glaucine all bind the $^9$PGQGXQ$^{13}$ motif, potentially blocking FabD substrates from entering the enzyme’s active site, however the antibacterial activity of boldine, dicentrine and glaucine, or the ability of these compounds to inhibit FabD, has also not been established (Rohini et al. 2012). Corytuberine was found to inhibit *E. tenella* FabD to a slightly higher degree than that of *H. pylori*, yet showed no significant MIC value (~650 µM) (Sun et al. 2012). However, *E. tenella* possesses both type I and II FAS pathways and thus may be able to utilise exogenous fatty acids, which could account for the poor MIC value (Lu et al. 2007, Sun et al. 2012). The lack of published, quantitative data regarding antimicrobial and inhibitory activities of these compounds presents an issue for the development of other FabD inhibitors, as there is no data to suggest that any such compounds would possess significant antimicrobial activity. Thus until a clear link between the inhibition of FabD and antimicrobial activity can be established, other FASII enzymes are more appropriate targets for inhibitor development.
CHAPTER 4 - Structural characterisation of the *Neisseria* FASII condensing enzymes FabH and FabF

4.1 Introduction

FabH, or β-keto ACP synthase III, catalyses the initial Claisen condensation reaction of the type II fatty acid synthesis pathway, typically combining acetyl-CoA and malonyl-ACP to produce the four carbon β-ketoacyl-ACP substrate for FabG (Figure 4.1). In a manner largely similar to FabH, FabF, or β-keto ACP synthase II, catalyses the condensation of two carbon atoms from malonyl-ACP onto an acyl-ACP primer. FabH substrate specificity is known to vary with some bacteria, such as *Bacillus subtilis* preferring branched chain acyl-CoA substrates, and *Mycobacterium tuberculosis* preferring long chain acyl-CoA substrates, to produce the mycolic acids and branched chain fatty acids found within their respective membrane compositions. However, FabH enzymes typically exhibit specificity for short chain thioesters (mainly C2-C4), and perform the initial condensation reaction of the FASII pathway. Subsequent elongation of the acyl chain to produce medium and long chain fatty acid components of the cell membrane is catalysed by FabF (Figure 4.1), which exhibits a much broader substrate specificity (Choi et al. 2000, Khandekar et al. 2001, Qiu et al. 2005, Gajiwala et al. 2009).

The question of whether FabH is an essential enzyme is contentious, Work by Yao et al. (2012) that demonstrates FabH is non-essential in wild type *E. coli* is in contrast to a previous study that demonstrated FabH to be an essential enzyme (Lai et al. 2003). Yao and colleagues (2012) suggest the previous studies utilised an *E. coli* strain harbouring *relA1* and *spoT1* alleles and the absence of FabH is lethal in combination with these mutant alleles. Genome wide transposon mutagenesis and metabolic modelling indicate
FabH is not essential in *N. meningitidis* (Mendum et al. 2011), yet due to conflicting results between the metabolic model and transposon data these results can not be considered conclusive. However, there is no contention that FabF is essential for bacterial viability in *N. meningitidis* (Mendum et al. 2011).

Despite the recent uncertainty in literature regarding the essentiality of these condensing enzymes, inhibitors have been designed against FabF, and FabH. Platencin, a potent inhibitor of both FabF and FabH, suggests the high degree of similarity between FabF and FabH active sites allows the design of inhibitors that can target both enzymes. Dual inhibitors could prevent both condensation reactions of the FASII pathway and thus the synthesis of necessary fatty acids, possibly resulting in pronounced antimicrobial activity against drug resistant *Neisseria spp.* However, the use of platencin and the related inhibitor platensimycin as therapeutic agents has proven problematic, with both compounds exhibiting poor antimicrobial activity against wild type *E. coli* due to efflux by the AcrAB–TolC efflux pump. Furthermore, while platensimycin exhibits little toxicity in HeLa cells it has been shown to strongly inhibit both the human and rodent FASI complex, and inhibit rodent liver fatty acid synthesis in *vivo* (Wu et al. 2011). While inhibition of FASI by platencin has not been reported, the structural similarity of these two inhibitors suggests platencin may also target the mammalian FAS complex. Despite this, platencin and platensimycin provide a proof of concept that dual FabH/FabF inhibitors can be potent broad spectrum antimicrobial agents. To facilitate the design of such inhibitors and gain further insight into the FASII pathway in *Neisseria spp.*, the three dimensional structures of *Neisseria meningitidis* FabF and FabH were determined by X-ray crystallography and compared to that of other bacteria, including those bound to known inhibitors.
4.2 Neisseria FabH

4.2.1 Structure determination

The gene encoding *Neisseria meningitidis* FabH (*NmFabH*) was amplified from genomic DNA and cloned into the expression vector pMCSG21 via ligation independent cloning (LIC) (sections 2.2.5-2.2.9). Spectinomycin resistant colonies were screened by PCR (section 2.2.10) to confirm the presence of *NmFabH*. Recombinant plasmid containing *NmFabH* was then isolated and sequenced (section 2.2.11) to ensure the fidelity of the clone. Recombinant *NmFabH* was expressed and purified as per sections 2.2.12 and 2.2.13, yielding concentrated protein of approximately 70 mg/mL (Figure 4.2).
Crystallisation trials were performed using *NmFabH* at neat and a 1:2 dilution with S200 buffer (section 2.2.13) with and without 5mM TCEP. Crystals were obtained in condition No. 1 of PEG/Ion (200 mM sodium fluoride, 20% PEG 3350) in the presence of 5 mM TCEP after approximately two months. Crystals were cryoprotected in 20% glycerol and flash cooled in liquid nitrogen. A total of 200° of X-ray diffraction data at 0.5° oscillations were collected at a wavelength of 0.9537 Å on the Australian synchrotron MX2 micro crystallography beamline. Data were integrated and scaled using iMosflm and Aimless to a resolution of 2.45 Å. The crystal was orthorhombic, displaying C222₁ symmetry, with the unit cell parameters \( a = 66.29 \), \( b = 96.36 \), \( c = 193.02 \) Å. Based on a molecular weight of ~33800 Da, two *NmFabH* molecules were estimated in the asymmetric unit, giving a solvent content of 46.15% and a Matthews coefficient of 2.60 Å³/Da. The structure of *NmFabH* was solved by molecular replacement using a monomer of *Burkholderia xenovorans* FabH (PDB:4DFE; Baugh et al. 2013) as the search model with an estimated two molecules within the asymmetric unit. Following model building and structure refinement as described in section 2.2.18, the final R and R-free values were 0.1992 and 0.2493 respectively (see Table 4.1 for details).
Figure 4.2 - Expression and purification of *N. meningitidis* FabH (*NmFabH*).

*NmFabH* was purified using a combination of affinity chromatography (A) and size exclusion chromatography (B). Pink line indicates the point of sample injection; blue line represents absorption in mAU; green line indicates the concentration of elution buffer (His buffer B). Protein expression and purification were visualised by SDS-PAGE (C), with samples loaded as follows: protein standards (i), whole cell extract (ii), cell lysate (iii), affinity column flowthrough (iv), affinity column elution (v), cleavage of affinity tag by TEV protease (vi), and concentrated *NmFabH* (vii). The single *NmFabH* crystal used for data collection (D). *NmFabH* crystals were grown in 200 mM sodium fluoride, 20 % PEG 3350, and 5mM TCEP. Collected diffraction data at 1° (E) and 90° (F) rotation.
Table 4.1 - *Nm*FabH diffraction data and model statistics.

<p>| | |</p>
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<tr>
<td>Unit cell angle (°)</td>
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<tr>
<td>Unique reflections</td>
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</tr>
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</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.

4.2.2 Overall structure of *Nm*FabH

The refined crystal structure of *Nm*FabH reveals a homodimeric $\alpha/\beta$ protein comprised of two tightly packed ~34 kDa monomers. This is consistent with other reports that FabH exists as a dimer in solution (Qiu et al. 2005, Alhamadsheh et al. 2007, Ramamoorthy et al. 2013), as well as the elution profile observed during size exclusion chromatography. Each monomer contains 10 $\alpha$-helices and 16 $\beta$-strands, and can be divided into two halves, an N-terminal domain (residues 1-171) and a C-terminal
domain (residues 172-320). These halves show a high degree of internal symmetry despite any significant sequence identity between the two domains. These two halves are related by a pseudo two-fold axis to form a thiolase fold characteristic of the thiolase superfamily, comprised of an $\alpha$-$\beta$-$\alpha$-$\beta$-$\alpha$ motif in which each $\alpha$ represents two $\alpha$-helices and each $\beta$ represents two 5 stranded mixed $\beta$-sheets. More specifically, this motif is comprised of $\alpha_4$-$\alpha_3$-$\beta_5$-$\beta_4$-$\beta_6$-$\beta_7$-$\beta_1$-$\alpha_5$-$\alpha_10$-$\beta_8$-$\beta_16$-$\beta_15$-$\beta_13$-$\beta_14$-$\alpha_7$-$\alpha_8$ in NmFabH (Figure 4.3), and comprises approximately two thirds of the protomer, with the remaining loop regions playing a role in substrate specificity and formation of the dimer interface.

The dimerisation interface of the NmFabH dimer comprises a buried surface area of $\sim 2630$ Å (20.2% of total surface area), with the most predominant dimerisation contacts occurring through secondary structure elements within the N-terminal domain. Alpha helix 4, and the loop preceding it interact with $\beta_8$ of the opposing monomer, and the $\alpha_5$ helices of each monomer cross at the dimer interface and form complimentary interactions with each other. Residues 186-207 form a hairpin loop that folds against its dimeric counterpart, and strand $\beta_5$, the outer strand of the N-terminal domain mixed $\beta$-sheet, is arranged antiparallel to its dimeric counterpart, creating a 10-stranded mixed $\beta$-sheet, which traverses the centre of the two molecules.

The active site, comprised of residues Cys114, His247 and Asn277 form the catalytic triad characteristic of Fab condensing enzymes and related thiolase family proteins (equivalent residues in E. coli FabH: Cys114, His244 and Asn274). The catalytic triad lies at the base of an L-shaped hydrophobic pocket, which accommodates binding of the acyl-ACP primer and malonyl-ACP. The entrance of the hydrophobic pocket is lined by residues 150-160, the N-terminal region of $\alpha_7$ and $\alpha_8$, the C-terminus of $\alpha_2$ (35-40), and
the loop region between β11 and β12. The catalytic triad is flanked by Trp33, Arg37, Arg151, Gly212, Pro213, Phe216, Lys217, and Arg252, which form the largely hydrophobic and electropositive substrate binding pocket. The acetyl-CoA primer binding site is largely formed by the side chains of Phe144, Phe159 and Leu191, while the acyl binding pocket is sandwiched between β13 and β15, and helices α7 and α8. Phe88 of the opposing monomer also forms a key component of the active site pocket, thus dimerisation of the two monomers is thought to be essential to FabH functionality.

4.2.3 Putative active site and reaction mechanism of NmFabH

The catalytic mechanism of FabH is well characterised in E. coli, and while the catalytic mechanism of NmFabH has not been demonstrated experimentally, sequence (Appendix 1) and structural homology indicate the catalytic mechanism of NmFabH is similar to that proposed for other FabH enzymes. FabH is thought to utilise a ping-pong mechanism to catalyse the Claisen condensation of the acyl-CoA primer and malonyl-ACP (Figure 4.4). The active site cysteine is essential for functionality, providing the attachment site for the acyl-intermediate, which is formed following nucleophilic attack of the terminal sulphhydryl on C2 of the incoming acyl-CoA primer, removing a single carbon from the acyl chain and releasing CO2. Originally thought to be activated by proton extraction through a water chain linked to His244, it is now clear that both His244 and Asn274 are too far from Cys114 to support hydrogen bonding (Davies et al. 2000, Qiu et al. 2005), and this is consistent in NmFabH.
Figure 4.3 - Tertiary and secondary structure elements of *N. meningitidis* FabH.

Two *NmFabH* monomers form a dimer, through interactions between the 5 stranded anti-parallel β-sheet of each molecule, forming a contiguous 10 stranded anti-parallel β-sheet that spans the biological unit (A). The secondary structure elements of *NmFabH* at 1° (B), 90° (C), and 180° (D) rotation. A 2D schematic of the *NmFabH* thiolase fold topology (E) and protein sequence of *NmFabH* (F). α-helices, β-strands, and loops are coloured yellow, cyan, and red, respectively.
Instead, the generation of a nucleophilic thionate ion occurs due to positioning of Cys114 at the N-terminus of α-helix α6, which results in a large helix dipole moment targeted towards the thiol, thus decreasing the pKa of the sulfhydryl. The amino groups of Cys114 and Gly309 form an oxyanion hole that accommodates the carbonyl oxygen of the acyl-CoA primer and the negative charge that arises during the tetrahedral transition state due to subsequent nucleophilic attack on the acyl-thioester. Following transfer of the acyl group to the terminal sulfhydryl of Cys114, CoA is released and malonyl-ACP binds to a charged hydrophobic region outside the active site pocket. The phosphopantetheine arm of malonyl-ACP extends into the hydrophobic tunnel, delivering the malonyl moiety to the catalytic residues. His244 and Asn274 form hydrogen bonds with the incoming malonyl moiety and deprotonate the enol intermediate, resulting in the formation of the carbanion on the C2 of malonyl-ACP thioester. The carbanion then attacks the acyl-ACP molecule and the resulting tetrahedral transition state is once again supported by the oxyanion hole formed by Cys114 and Gly309, the transition state is then resolved, releasing β-ketoacyl-ACP (Davies et al. 2000, Qiu et al. 2001, White et al. 2005) (See Figure 4.4 for an overview).
4.2.4 Putative *NmFabH* Co enzyme A and ACP binding sites

Soaking and co-crystallisation experiments with *NmFabH* failed to yield crystals containing bound CoA. However, due to the conserved nature of the catalytic triad, and
substrate bindings site, substrate interactions can be somewhat inferred through superposition of EcFabH structures in complex with CoA. Superposition of *E. coli* (EcFabH; PDB:1EBL/1HND) complex with CoA indicates the adenine moiety of CoA interacts with Trp33 of helix α2 and Arg153 of helix α5 at the entrance to the active site tunnel. The diphosphate group likely forms hydrogen bonds with the basic side chains of Arg37, Leu158, Met210 and Arg251, while hydrogen bonds between the pantetheine arm and residues Phe144, Phe159, Gly212 and Asn277 anchor the moiety in the active site pocket adjacent to the active site cysteine (Figure 4.4). It is unknown if FabH interacts with the 3′-phosphate of CoA, Qiu et al. (2001) suggest the oxygen atoms of the phosphate interact with adjacent solvent molecules, while the *NmFabH:CoA* model generated through superposition indicates the 3′-phosphate molecule could be stabilised by Arg153.

Differences within the loop regions and base of the primer binding site are thought to provide the basis for differing substrate specificities between FabH enzymes. Gajiwala et al. (2009) suggest that while the amino acid sequence of this region varies, structural differences are due to different rotamer conformations rather than amino acid substitutions or inserts observed within these regions. Furthermore, Gajiwala et al. (2009) suggest enzymes that utilise branched chain fatty acids adopt a conserved rotamer conformation, equivalent to that of Phe298 in *S. aureus* FabH (*SaFabH*; PDB:3IL7), Phe312 in *E. faecalis* FabH (*E/FabH*; PDB:3IL4) and Tyr304 in *Mycobacterium tuberculosis* FabH (*MtFabH*; PDB:1M1M), where the aromatic ring of Phe/Tyr is rotated inward toward the active site, causing a reduction in the size of the hydrophobic pocket. This is not consistent with the structures of *EcFabH* and *Haemophilus influenza* FabH (PDB:3IL3) structures, in which the side chains of the equivalent residues, Phe304 and Phe303 respectively, rotate away from the active site.
towards the dimer interface. Although possible, how the differing conformations of Phe/Tyr residues increases the specificity for branched chain fatty acids, if at all, is unclear.

Structural comparison reveals the proposed binding pocket of SaFabH to be larger than that of EcFabH due to movement of the several residues lining the primer binding site. Residues Phe157 and 190-199 show considerable movement away from the active site. Residues Phe87 and Leu142 that flank the active site also show pronounced shifts (1.7 Å and 3.0 Å respectively), extending the primer binding pocket to accommodate both straight and branched chain acyl-CoA primers. These changes are similar to that observed in MtFabH, in which Phe87 and Arg196 of SaFabH and EcFabH are replaced by threonine and isoleucine, elongating the primer binding pocket to accommodate 16 carbon acyl chains. This clearly indicates that these regions play a major role in determining the size of the primer binding cleft, and therefore likely to have the greatest impact on substrate specificity. Many of these residues differ in NmFabH, with the pair of Phe87 and Leu142 in EcFabH replaced by Phe88 and Phe144 in NmFabH. Structurally, this substitution does not appear to increase the size of the primer binding domain beyond that of E. coli, and is unlikely to alter substrate specificity. The acyl binding cleft of NmFabH appears to be extended by approximately 5 Å in length compared to that of E. coli FabH, due to the replacement of Phe304 in E. coli with isoleucine in N. meningitidis. Otherwise, the binding pocket mirrors the size and shape of E. coli, which is consistent with the predominantly straight chain fatty acid composition observed in Neisseria spp. (Rahman et al. 2000).

The mechanisms through which FabH binds to ACP has not been clearly established. However, CoA and ACP share the same pantetheine arm, thus it is likely the
interactions between FabH and these two moieties are highly similar, yet the precise interactions at the interface of ACP and the surface of FabH are not known. The surface of *Nm*FabH surrounding the active site tunnel is hydrophobic and electropositive, ideally suited to bind the acidic ACP. To investigate the role of ionic interactions between ACP and this region, Zhang et al. (2001) removed the basic side chains of lysine residues 214, 256, and 257, and Arg249 from *Ec*FabH, effectively eliminating the electropositive charge of this area. Mutation of lysine residues 214, 256, and 257, and Arg249 to alanine residues significantly reduced FabH:ACP complex formation. Complex formation was further reduced by the replacement of these residues with glutamate. These mutations did not impair CoA binding compared to that of wild type, clearly confirming that the ACP binds to FabH at this location.

### 4.2.5 Potential *Nm*FabH interactions with platencin and platensimycin

Both platencin and platensimycin display significant inhibitory activity against FabH, with IC$_{50}$ values of 16.2 µM and 67 µM against *Sa*FabH. Cerulenin and thiolactomycin are poor inhibitors of FabH due to differences within the active site catalytic triad and hydrophobic binding pocket (Price et al. 2001). In contrast, it is unclear why there is ~4 fold difference in susceptibility between platencin and platensimycin, in part due to the lack of experimentally determined structures of FabH in complex with either of these inhibitors. Docking of platencin and platensimycin into the active site of *Ec*FabH by Jayasuriya and co-workers (2007) show the terminal carboxylic acid of platencin and platensimycin forms hydrogen bonds with residues Asn274 and His244 of the *Ec*FabH catalytic triad and His303 and His310 of the *Ec*FabF catalytic triad. The only difference between the two inhibitors are the cyclic rings of the ketolide groups posed at the entrance of the active sites, with platensimycin containing a pentacyclic ketolide with an ether ring, and platencin containing a tetracyclic motif without the ether ring.
(Jayasuriya et al. 2007) propose the ether of the platensimycin pentacyclic ring is opposed by the non-polar residues Trp32, Ile155, and Ile156, while the tetracyclic ring of platencin makes favourable hydrophobic interactions with these residues. Docking of platencin and platensimycin into the active site of \( \text{NmFabH} \) reveals a similar conformation to that reported by (Jayasuriya et al. 2007) within the hydrophobic substrate binding pocket, preventing the phosphopantetheine moiety of CoA or ACP from binding (Figure 4.4), indicating these compounds could prove to be useful antimicrobial agents or lead compounds.

4.3 \textit{Neisseria} FabF

4.3.1 Structure determination

The gene encoding \textit{N. meningitidis} FabF (\( \text{NmFabF} \); No. YP_974338.1) was cloned into the expression vector pMCSG21 via LIC (sections 2.2.5-2.2.9). Recombinant \( \text{NmFabF} \) was then overexpressed in BL21(DE3)plysS cells and purified as per sections 2.2.12 and 2.2.13. Initial crystallisation of \( \text{NmFabF} \) was performed via hanging drop vapour diffusion technique using the commercially available sparse matrix crystal screens outlined in section 2.2.17. Clusters of tightly packed hexagonal shaped crystals were obtained in condition No. 21 of PEG/Ion (200 mM sodium formate, 20% PEG 3350) after approximately 2 weeks. To obtain large individual crystals, crystallisation conditions were optimised by varying sodium formate and PEG 3350 concentrations. The crystal used for structure determination was obtained in 250 mM sodium formate, 20% PEG 3350. Prior to data collection, optimised crystals were cryoprotected in 25% glycerol and flash cooled in liquid nitrogen. A total of 180° of X-ray diffraction data were collected on the Australian synchrotron MX2 micro crystallography beamline at 0.5° oscillations. Data were integrated and scaled to a resolution of 2.10 Å as per
section 2.2.18. The \textit{Nm}FabF crystal belonged to the primitive hexagonal space group P6\textsubscript{5}, with the unit cell parameters \(a = 153.49\), \(b = 153.49\), \(c = 66.47\) Å.

Two molecules of \textit{Nm}FabF (each monomer 43 kDa) were expected in the asymmetric unit, with a solvent content of 53.22\% and a Matthews coefficient of 2.63 Å\textsuperscript{3}/Da. This was confirmed by the placement of a dimer within the asymmetric unit by molecular replacement (section 2.2.18) using \textit{E. coli} FabF (PDB:2GFW; Wang et al. 2006) as a search model. Initial model building was performed using Phenix autobuild, subsequent model building and refinement was performed using WinCoot and Phenix refine as described in section (section 2.2.18) (see Table 4.2 for diffraction data and model statistics), producing a final model with a R and R\textsubscript{free}, values of 0.199 and 0.249, respectively.

### 4.3.2 Overall structure of \textit{Nm}FabF

The crystal structure of \textit{Nm}FabF contains two monomers within the asymmetric unit, forming a homo-dimer. \textit{Nm}FabF is an \(\alpha/\beta\) protein, in which each subunit is comprised of 12 \(\alpha\)-helices and 14 \(\beta\)-strands. Like FabH, FabF contains a thiolase fold characteristic of that superfamily. The core structure of this fold consists of two \(\beta\) \(\alpha\) \(\beta\) \(\alpha\) \(\beta\) \(\beta\) motifs related by pseudosymmetry, in which a 5 stranded mixed \(\beta\)-sheet is flanked by two \(\alpha\)-helices one side and a single \(\alpha\)-helix on the other, with the topology \(\alpha5-\alpha3-\beta5-\beta4-\beta6-\beta7-\beta1-\alpha6-a12-\beta8-\beta14-\beta13-\beta9-\beta10-\alpha9-\alpha10\) (Figure 4.5). The insertion of small \(\alpha\)-helices, \(\beta\)-strands and 3-10 helices do not disrupt the overall topology of the thiolase fold, instead arranging into lobes outside this motif that aid in dimerisation of the monomers. The dimerisation interface encompasses a large surface area of the \textit{Nm}FabF monomers, with \(~18.4\%\) of the total surface area (~156230 Å\textsuperscript{2}) inaccessible due to dimerisation. The interactions within the dimer interface include an extensive array of salt bridges and
polar interactions. Noticeably the residues 160-162 of β5 are arranged anti-parallel to the same region of the opposing monomer, resulting in the combination of the N-terminal β-sheets in each protomer to form a continuous 10 stranded mixed β-sheet that spans the centre of the dimer interface, similar to that observed in NmFabH. Other major interactions occur through secondary structure elements outside the thiolase fold; the loop between strand β4 and helix α4 (residues 108–112) interacts with residues of helix α7, and helix α4, of the opposing subunit. Additional interactions are made by residues from helix α5, which pack against the C-terminal end of β-strands β8 and β14.

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<th>Table 4.2 - NmFabF diffraction data and model statistics.</th>
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Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.
Figure 4.5 - Tertiary and secondary structure elements of *N. meningitidis* FabF.

Two *NmFabF* monomers dimerise through interactions between the 5 stranded anti-parallel β-sheets of each monomer, forming a contiguous 10 stranded anti-parallel β-sheet similar to that observed in *NmFabH* (A). The secondary structure elements of *NmFabF* at 1° (B), 90° (C), and 180° (D) rotation. A 2D schematic of the *NmFabF* thiolase fold topology (E), and protein sequence of *NmFabF* (F). α-helices, β-strands, and loops are coloured red, yellow, and green, respectively.
4.3.3 Putative *NmFabF* active site and reaction mechanism

Similar to that of *NmFabH*, the active site of FabF contains a catalytic triad consisting of a central cysteine and two histidine residues (Cys165, His303, His340) (Figure 4.6). The catalytic triad sits at the base of a curved hydrophobic pocket, which facilitates the binding of the growing acyl chain. The active site cysteine is located at the N-terminus of helix α8. His303 and His340 lie 3.75 and 3.54 Å from the Cys165 on the C-terminal end of strand β9 and the loop between helices α11 and α12, respectively. In addition to the active site triad, the hydrophobic pocket is further lined by residues 200-204 at the C-terminus of helix α7, residues 225-230 of the loop connecting α7 and β7, residues 270-280 of the loop linking β8 and α9, N-terminal residues of α9 (residues 278-284), residues 295-310 of β9, the loop preceding helix α10 (residues 307-318), residues 398-404 spanning the C-terminal end of β13, and the N-terminal region of β14. Dimerisation is essential to the formation of the active site, with residues 132-142 of the adjacent monomer completing the wall of the hydrophobic pocket.

Based on analogy with other FabH enzymes (Appendix 1) the condensation reaction of *NmFabF* is thought to occur via a two-step mechanism in which an acyl group from an acyl-ACP molecule is transferred to the active site cysteine, the FabF bound intermediate is then transferred to an incoming malonyl-ACP molecule. The position of Cys165 at the N-terminal end of a long α-helix (α6) results in a strong dipole moment targeted towards the thiol, decreasing the pKa of the sulfhydryl. This leads to the generation of nucleophilic thiolate, which initiates a nucleophilic attack on the terminal thiol of the bound acyl-ACP, resulting in the transfer of the substrate to the active site cysteine, and displacing ACP. In conjunction with the active site cysteine, the conserved residues Phe229 and Phe401 form an oxyanion hole, which accommodates the carbonyl oxygen of the bound acyl chain and acts to stabilise the negative charge on the carbonyl
oxygen of the acyl thioester during the tetrahedral transition state. The incoming malonyl-ACP binds to the acyl-enzyme intermediate and the active site residue His340 donates a hydrogen bond to the carbonyl group oxygen of C1 of the malonyl-ACP, forming a second oxyanion hole. The other active site histidine (His303) deprotonates a conserved water, initiating a nucleophilic attack on the C3 of the malonate, thus releasing bicarbonate. The oxyanion hole formed by His340 acts to stabilise the acyl chain carbonyl oxygen during the tetrahedral transition state following the nucleophilic attack, promoting the formation of a carbanion at the C2 of the bound malonate. The carbanion that is generated at position C2 of the bound malonate performs a nucleophilic attack on the C1 of the acyl-intermediate linked to the active site cysteine. The Cys165/Phe401 oxyanion hole stabilises the formation of another tetrahedral intermediate which collapses to expel the cysteine thiol, releasing enzyme and an acyl-ACP elongated by two carbons (Zhang et al. 2006) (Figure 4.6).

4.3.4 Potential NmFabF substrate interactions and ACP binding site

Analysis of the active site pocket of NmFabF revealed that the entrance to the active site is partially blocked by Phe401, thus preventing access to Cys165. Superimposition of structures of E. coli FabF in complex with lauric acid and the inhibitor cerulenin onto the structure of apo-NmFabF shows this residue rotates to accommodate substrate binding. In comparison to the structure of EcFabF bound to lauroyl-CoA and cerulenin, the aromatic ring of Phe401 in the unbound NmFabF crystal structure protrudes into the malonyl-ACP binding site, “closing” this part of the active site (Figure 4.7). Seemingly, this is to prevent the binding of malonyl-ACP before transacylation of the acyl-ACP chain and the formation of the acyl-enzyme intermediate, thus ensuring that the forward reaction proceeds to completion.
The formation of the tetrahedral transition state reaction intermediate following transacylation of the acyl-ACP chain is believed to cause the aromatic ring of Phe400 to rotate away from the active site, “opening” the malonyl-ACP binding site, and allowing the substrate to access the acyl-enzyme intermediate. Structural and functional characterisation of the *Streptococcus pneumoniae* FabF (SpFabF) mutants support the hypothesis that Phe400 acts as a “gatekeeper” to control the order of substrate binding. SpFabF mutants H303A and F396A (equivalent residue Phe401 in NmFabF) display reduced condensation activity compared to the wild type (Zhang et al. 2006).

**Figure 4.6 – The FabF reaction mechanism.** The catalytic mechanism for the FabF elongation reaction, as proposed by Zhang et al. (2006). First, an acyl group from acyl-ACP is transferred to the active site cysteine. An oxyanion hole formed by residues Phe229, Phe401, and the active site cysteine accommodates the negative charge on the acyl chain carbonyl oxygen that develops during the transition state, and ACP is released. The FabF:intermediate complex is then bound by malonyl-ACP. His303 then activates a conserved water, initiating a nucleophilic attack on the C3 of the malonyl group. The oxyanion hole formed by His340 acts to stabilise the acyl chain carbonyl oxygen during the tetrahedral transition state following the nucleophilic attack, and promotes the formation of a carbanion at the C2 of the bound malonate. The carbanion attacks the acyl-intermediate linked to the active site cysteine. The Cys165/Phe401 oxyanion hole stabilises the formation of another tetrahedral intermediate which collapses to expel the cysteine thioester, releasing enzyme and an acyl-ACP elongated by two carbons. Figure adapted from Zhang et al. (2006).
The formation of triacetic acid lactone (TAL), a product produced via the repeated condensation and transacylation of malonyl-ACP due to reverse order substrate binding, is also significantly greater than the condensation activity in these mutants. The crystallographic structure of SpFabF H303A reveals that removal of the imidazole ring from His303 results in the absence of the structured waters once linked to His303 via hydrogen bonding. Reduced electron density for the Phe396 (equivalent residue Phe401 in NmFabF) side chain of this structure compared to that of wild type SpFabF indicates the absence of these structured waters allows free rotation of Phe396, thus allowing malonyl-ACP to bind prior to the formation of the acyl-enzyme intermediate, resulting in the formation of TAL rather than elongated β-ketoacyl-ACP (Zhang et al. 2006).

Although there are no published structures of FabF:ACP complexes available to determine the mechanism by which ACP interacts with FabF superposition of EcFabH in complex with CoA and malonyl-CoA onto the structure of NmFabF suggests the pantetheine moiety may interact with a number of highly conserved residues, including Lys208, Ala209, Asp229, Gly230, Phe231, Thr305, Thr307 and Phe401. Lys208, Ala209, Asp229, Gly230, and Pro308 line the entrance to the acyl-ACP binding site and may interact with ACP and the base of the phosphopantetheine arm. Residues that line the active site cleft include Phe231, Thr305, Thr307, and Phe401. Residues Thr305 and Thr307 are positioned to form hydrogen bonds with the peptide bonds of the phosphopantetheine moiety similar to that of Asp277 of EcFabH, while Phe231 and Phe401 appear likely to form hydrophobic interactions with the carbon atoms towards the terminal sulphur atom of the molecule. Superposition of FabH structures in this manner allows us to predict likely interactions for the ACP phosphopantetheine moiety, however such predictions are purely theoretical until confirmed by mutagenesis or a structure of FabF in complex with ACP is available.
Figure 4.7 - Superposition of FabF inhibitors within the active site of *N. meningitidis* FabF. (A-D) The catalytic triad of *Nm*FabF is comprised of Cys165, His303 and His340, superposition of the FabF inhibitors platencin (green), platensimycin (cyan), thiolactomycin (blue), demonstrates these inhibitors block entrance to active site of FabF. The inhibitor cerulenin (pink) binds in the pocket which accommodates fatty acid intermediate (orange:dodecanoic acid). (C) Like *Nm*FabH, the catalytic triad of *Nm*FabF sits at the base of a hydrophobic pocket (as indicated by red surface). (D) Superposition of apo *E. coli* FabF (pink; PDB: 2GFW) and *E. coli* FabF in complex with platencin (green; PDB: 3H02) and platensimycin (cyan; PDB: 2GFX) reveal Phe401 adopts a similar “closed” conformation to that of apo *E. coli* FabF, the binding of platencin or platensimycin mimic that of a substrate, causing Phe401 to rotate away from the active site into an “open conformation”.
4.3.5 *NmFabF* interactions with platencin and platensimycin

The inhibitors platencin and platensimycin are of great interest due to their unique ability to inhibit both FabF and FabH condensing enzymes, as well as their enhanced potency compared to the other FabF inhibitors, cerulenin, and thiolactomycin. In contrast to FabH, FabF shows the greatest sensitivity to platensimycin, with an IC$_{50}$ value of 48 nM against *SaFabF* compared to 0.113 μM for platencin (Wang et al. 2006, Wang et al. 2007). Furthermore, the structures of *EcFabF* in complex with platencin and platensimycin have been solved, revealing the binding mechanism for these two inhibitors. Comparison of platensimycin and platencin bound *EcFabF* structures to wild type *EcFabF* and *NmFabF* show the “gatekeeper” residue Phe400 (*NmFabF* equivalent Phe401) rotates into the open conformation edge to face against the benzoic acid ring of platencin and platensimycin, while the carboxylic acid group forms hydrogen bonds with the catalytic Histidines, His303 and His340. The rotation of Phe400 is necessary to avoid a steric clash with the benzoic acid ring of the inhibitors, thus platensimycin and platencin selectively bind FabF after acylation of the catalytic cysteine (Cys165). The amide group positioned between the benzoic acid and ketolide moieties of platencin and platensimycin interacts with Thr270, Thr307 and Ala309 (*NmFabF* equivalent residues; Thr272, Thr307 and Leu309) (Wang et al. 2006, Wang et al. 2007, Singh et al. 2009). The ketolide moieties of the two inhibitors are positioned at the entrance of the hydrophobic binding site in both *EcFabF* and *NmFabF*, as observed in FabH docking studies, and the ether oxygen of the platensimycin pentacyclic ring makes a hydrogen bond to Thr270 of *EcFabF*. The tetracyclic ring of platencin does not possess an ether cyclic ring. As such, this interaction is missing in the *EcFabF/platencin* complex, with the lack of this hydrogen bond resulting in a reduced affinity to FabF (Figure 4.7) (Jayasuriya et al. 2007). However, the loss of this ether group is also attributed to a

The unique ability of these two molecules to inhibit both FabF and FabH at low concentrations with low toxicity demonstrates a clear potential as lead compounds for the development of therapeutic agents, however the antimicrobial efflux and interactions with the mammalian FASI complex occlude the use of these molecules as therapeutic agents (Wu et al. 2011). Efflux of these molecules may not be an issue in *N. meningitidis* and *N. gonorrhoeae* due to differing efflux pumps to *E. coli* (Rouquette-Loughlin et al. 2003, Kamal et al. 2007, Kamal et al. 2010), and structural based drug design techniques in conjunction with careful comparison of the structural similarities between the mammalian FabF and FabH homologs could lead to potent broad spectrum antimicrobials.
CHAPTER 5 - Structural characterisation of the *Neisseria* FASII reductases FabG and FabI

5.1 Introduction

The reduction reactions of the FASII pathway in *Neisseria* *spp.* are catalysed by two enzymes that belong to the short chain dehydrogenase family (SDRs), and share similar structural motifs, active sites, and catalytic reaction mechanisms. FabG or β-keto ACP reductase, catalyses the NADPH dependent reduction of β-ketoacyl-ACP intermediates to β-hydroxyacyl-ACP products, the first of two reduction reactions within the FASII pathway (Figure 5.1). FabG is an essential enzyme, highly conserved and ubiquitously expressed within most bacteria as a single isoform, making it a potential target for new broad spectrum antimicrobial agents (Zhang et al. 1998, Marrakchi et al. 2002, Lai et al. 2004). A number of inhibitors have been identified including chlorogenic acid, various cinnamic acid derivatives, and some green tea catechins such as epigallocatechin gallate (EGCG). Whilst the IC₅₀ values of such inhibitors indicate a strong binding affinity for FabG, many of these compounds possess poor minimum inhibitory concentration values against *E. coli* and *S. aureus*, making their use as therapeutic agents limited due to bacterial efflux systems (Zhang et al. 2004, Li et al. 2006a, Kristan et al. 2009, Matsumoto et al. 2012). That these efflux pumps are not present in *Neisseria* *spp.* provides an exciting opportunity to characterise the structural basis of these interactions, and provide a possible platform for structure based drug design.

The second reductase reaction, and final reaction of the FASII cycle, is catalysed by FabI or enoyl-ACP reductase. FabI catalyses the NADH dependent removal of the C2:C3 *trans* double bond of trans-2-enoyl-acyl-ACP, the product of the dehydration reaction catalysed by FabZ, forming a saturated acyl-ACP (Figure 5.1). As an essential
protein in many bacteria, and the major rate-limiting step of the FASII pathway, FabI has been the focus of many drug development programs and is the primary target of the widely used inhibitor triclosan (Baldock et al. 1996, McMurry et al. 1998, Stewart et al. 1999, Singh et al. 2011). Whilst some developed inhibitors that display very high affinity for FabI, such as AFN-1252, MUT056399 and CG400549, have entered into clinical trials, single amino acid substitutions within the drug binding site have been reported to confer resistance (Park et al. 2007, Escaich et al. 2011, Yao et al. 2013). Triclosan resistant bacteria have also been reported, due to point mutations in their FabI genes (Webber et al. 2008, Copitch et al. 2010), indicating a need for new antimicrobials more resilient to single point mutations.

EGCG, a green tea catechin related to chlorogenic acid, has been shown to inhibit the FabG and FabI enzymes of E. coli, and the malarial parasite Plasmodium falciparum (Zhang et al. 2004, Tasdemir et al. 2006, Matsumoto et al. 2012). While EGCG itself possesses lower antimicrobial activity than other FabI inhibitors, EGCG-based inhibitors designed to enhance interactions within the EGCG binding sites of both FabI and FabG may yield potent antimicrobials, which target the two reductases simultaneously, possibly reducing the extent to which point mutations could confer antimicrobial resistance. As such, X-ray crystallography was used to determine the structure of Neisseria meningitidis FabG and FabI enzymes and investigate their potential as targets for combating emerging drug resistance in N. gonorrhoeae and N. meningitidis. This chapter will discuss the structures of these enzymes and the structural basis of resistance conferring mutations.
Figure 5.1 - The roles of FabG and FabI in the FASII pathway of *Neisseria spp.*
The reduction reactions of the FASII pathway in *Neisseria spp.* are catalysed by FabG, which catalyses the NADPH dependent reduction of β-ketoacyl-ACP intermediates to β-hydroxyacyl-ACP products, and FabI, which catalyses the NADH dependent reduction of trans-2-enoyl-acyl-ACP, the product of the dehydration reaction catalysed by FabZ, to produce a fatty acyl-ACP molecule.

5.2 *Neisseria* FabG

5.2.1 Structure determination

The *fabG* gene from *N. meningitidis* (Gene ID: 4676438) was amplified from genomic DNA and cloned into the expression vector pMCSG21 as described in sections 2.2.5-2.2.10 and previous chapters. Recombinant NmFabG was expressed and purified as per sections 2.2.13 and 2.2.14 respectively, and concentrated to approximately 60 mg/mL (Figure 5.2).
Figure 5.2 - Expression and purification of *N. meningitidis* FabG (*NmFabG*).

*NmFabG* was expressed in *E. coli* BL21(DE3)pLysS cells and purified by a combination of affinity chromatography (**A**) and size exclusion chromatography (**B**). Pink line indicates the point of sample injection; blue line represents absorption in mAU; green line indicates the concentration of elution buffer (His buffer B). *NmFabG* expression and purification were assessed by SDS-PAGE (**C**), with samples loaded as follows: protein standards (i), whole cell extract (ii), cell lysate (iii), affinity column flowthrough (iv), affinity column elution (v), cleavage of affinity tag by TEV protease (vi), size exclusion chromatography fractions (vii-ix), and concentrated *NmFabG* (x). The *NmFabG* crystal used for data collection mounted in cryoprotectant at 1° (**D**) and 90° (**E**) rotation, and collected diffraction data at 1° (**F**) and 90° (**G**) rotation.
Crystallisation trials were performed using recombinant *NmFabG* at concentrations of ~60 mg/mL, 30 mg/mL and 15 mg/mL (see section 2.2.17). Clusters of plate shaped crystals were obtained in condition No. 54 of the Hampton Research PEG/Ion screen (200 mM ammonium phosphate dibasic, 20% PEG 3350) overnight. To reduce the formation of clusters and isolate a single diffraction quality crystal, *NmFabG* was screened against a range of PEG 3350 and ammonium phosphate concentrations, with and without 10% glycerol. Microseeding was also used to increase the number of potential nucleation sites. Crystals grown in 100-250 mM ammonium phosphate dibasic, 20% PEG 3350 and 10% glycerol were harvested by pipette, diluted in reservoir solution, pulverised into microseeds by vortex mixer, and freeze thawed for one cycle before 1 µL of this seed stock was added to 80 µL of concentrated protein. Serial seeding using these conditions resulted in the formation of diffraction quality crystals in 125 mM ammonium phosphate dibasic, 20% PEG 3350 and 10% glycerol after two rounds of microseeding (Figure 5.2)

Crystals were cryoprotected in 125 mM ammonium phosphate dibasic, 22% PEG 3350 and 25% glycerol, and flash cooled in liquid nitrogen after 5 days. One hundred and eighty degrees of X-ray diffraction data were collected at 0.5° oscillations at a wavelength of 0.9537 Å on the Australian Synchrotron MX2 micro crystallography beamline. Data were integrated in XDS, then merged and scaled using Aimless to a resolution of 1.7 Å. The large plate-like crystal displayed P2₁2₁2₁ symmetry, with the unit cell parameters \(a = 66.85\), \(b = 112.13\), \(c = 121.68\) Å. Based on a molecular weight of 26 kDa, a solvent content of 43.93% and a Matthews coefficient of 2.19 Å³/Da, four *NmFabG* molecules were expected in the asymmetric unit. The structure of *NmFabG* was solved by molecular replacement using a monomer of *EcFabG* (PDB:1I01; Price et al. 2001) as a search model, with an estimated four molecules within the asymmetric
unit. Following model building and structure refinement as described in section 2.2.18, the final R and R-free values were 0.155 and 0.178 respectively (See Table 5.1 for crystallographic and data collection statistics). Attempts to obtain a structure of NmFabG in complex with its co-factor NADPH by either co-crystallisation or soaking failed to yield a structure containing the ligand when using crystallisation conditions based on those of apo NmFabG. Therefore, new crystallisation screens were undertaken to find conditions that would induce crystallisation in the presence of NADPH.

To obtain crystals of NmFabG:NADPH complex, recombinant NmFabG was mixed with NADPH at a molar ratio of 1:10 respectively and screened against the Hampton Research and Molecular Dimensions crystallisation kits as per apo NmFabG. Initial crystals were observed in conditions containing 4-8% Tacsimate, 12-20% PEG and a pH ranging from 5.0 to 8.0. These crystals were optimised by varying the concentration of PEG and a Tacsimate substitute (Table 2.6), as well as pH, in conjunction with microseeding as conducted for apo NmFabG crystals. NmFabG:NADPH crystals collected for X-ray diffraction data appeared overnight in 8% Tacsimate substitute at pH 7.0 and 18% PEG 3350, using microseed stock of apo NmFabG. NmFabG:NADPH crystals were grown for 4 days then cryoprotected in 8% Tacsimate substitute, 20% PEG 3350 and 25% glycerol at pH 7.0, and flash cooled in liquid nitrogen. X-ray diffraction data of NmFabG:NADPH crystals were collected using the Australian Synchrotron MX2 micro crystallography beamline at 0.5° oscillations for 360° of rotation, and a wavelength of 0.953 Å. Data were processed as per apo NmFabG and scaled to a resolution of 2.20 Å. The crystals belonged to the space group P1, with 16 NmFabG molecules within the asymmetric unit, as confirmed by molecular replacement using a monomer of apo NmFabG as a search model. NADPH molecules were placed using a combination of Phenix ligand fit, superposition with EcFabG in complex with
NADPH, and the NCS ligands function in WinCoot. NADPH molecules were further modelled to fit the electron density in WinCoot. In total, 16 NADPH molecules were observed in the asymmetric unit, with a single molecule bound to each NmFabG monomer. Final model, crystal, and data collection statistics are all listed below in Table 5.1.

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Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.

5.2.2. Overall structure of NmFabG and NmFabG:NADPH models

The crystal structure of apo NmFabG contained four monomers of approximately 26 kDa in the asymmetric unit, arranged as a homo-tetramer. Each monomer contains a
nucleotide binding Rossmann fold motif comprised of a seven stranded parallel β-sheet flanked by eight α-helices with the topology β1-α1-β2-α2-β3-α3-β4-α4-β5-α5-β6-α6-α7-α8-β7. Helices α6 and α7 are arranged in a helix-turn-helix structure separated from the centre of the Rossmann fold, giving rise to a flexible domain that forms the lid of the active site pocket. Helix α8 and strand β7 rejoin the Rossmann fold, with β7 arranging parallel to β6, and α8 aligned parallel to these two β-strands (Figure 5.3).

The four molecules of the NmFabG homo-tetramer are related by D2 symmetry, forming two types of dimer interfaces. The dimer interface that lies between chains A and B (A/B interface) and chains C and D (C/D interface) is comprised largely of interactions involving helix α8, which interacts with the loop regions between the N-terminus and β1, α6 and β6, α7 and α8, β7 and the C-terminus of the opposing monomer. Additional contacts are made by the C-terminal end of helix α5 and the loop regions between α7 and α8, and β7 and the C-terminus of the opposing chain, with ~11% (~1616 Å²) of the total surface area buried due to this dimerisation interface. The second dimerisation interface, formed between monomers A and D (A/D interface) and the equivalent between chains B and C (B/C interface) envelopes ~11% (~1584 Å²) of the total surface area. The interface is comprised of interactions between α4, and helices α4’ and α5’ of the opposing monomer, and interactions between α5, α5’ and the loop between α5-β5 of the opposing monomer.
Figure 5.3 - Tertiary and secondary structure elements of *N. meningitidis* FabG.

*Nm*FabG forms a homo-tetramer related by D2 symmetry, with two distinct dimer interfaces. One interface is formed primarily through interactions between helices $\alpha_4$ and $\alpha_5$ of two monomers, with the second formed through the 7 stranded $\beta$-sheets of two monomers, that align parallel to that of the opposing monomer, forming a 14 stranded $\beta$-sheet that spans two *Nm*FabG molecules (A). The secondary structure elements of *Nm*FabG at 1° (B), 90° (C) and 180° (D) rotation. A 2D schematic of the *Nm*FabG topology (E) and protein sequence of *Nm*FabG (F). $\alpha$-helices, $\beta$-strands, and loops are coloured cyan, red, and magenta, respectively.
The biological unit of FabG enzymes have been reported as both dimers and tetramers in varying species, while some reports indicate a concentration dependent tetramerisation (Marrakchi et al. 2002, Lai et al. 2004, Wickramasinghe et al. 2006, Dutta et al. 2012). The essentiality of dimer or tetramer formation has not been established, however recent development of inhibitors, which bind at the dimer interfaces within the FabG tetramer, indicate tetramer formation may induce conformational changes necessary for biological function. As inhibitors targeting the dimer interfaces of FabG may be useful in combating drug resistance in Neisseria spp. and reports of FabG biological units vary, we used a combination of small angle X-ray scattering (SAXS) and gel filtration to determine the relevant biological oligomeric state of NmFabG. Elution from the gel filtration revealed a native molecular weight of approximately 100 kDa, corresponding to a tetramer. This was further supported by SAXS measurements, which all showed a greater correlation to the tetrameric state than monomeric or dimeric assemblies (Figure 5.4).

5.2.3 Putative NmFabG active site architecture and reaction mechanism

FabG catalyses the reduction of β-ketoacyl-ACP intermediates at the C3 position to produce β-hydroxyacyl-ACP (Figure 5.5). Similar to other SDRs, the active site of FabG is comprised of a Ser-Tyr-Lys catalytic triad located within the NADPH binding cleft of a Rossmann fold motif, a mechanism which has also been characterised in E. coli (Price et al. 2001, Price et al. 2004, White et al. 2005). The active site residues identified in EcFabG are conserved in NmFabG and other FabG proteins, thus the active site and reaction mechanism can be largely conferred through homology. The active site residue Ser138 resides in an ordered loop region between β5 and α5, while residues Tyr155 and Lys159 are located on the C-terminal end of α5.
The reaction is likely to proceed via an ordered reaction mechanism in which NADPH first binds, inducing a conformational change causing the active site residues to shift...
into an active conformation. Upon binding NADPH the active site residues Tyr155 and Lys159 rotate towards the nicotinamide ribose of the co-factor to form hydrogen bonds with the 2’ hydroxyl, and 2’- and 3’-hydroxyl groups of the ribose, respectively. The hydroxyl group of Ser142 rotates into a hydrophobic binding pocket lined by residues Val155, Val156, Ile159, Phe199, Ile216, and Ile258, which accommodates the ACP-linked fatty acid intermediates. Superposition of the structure of NmFabG and FabG from Bacillus sp. strain SG-1 in complex with co-factor and the substrate analogue acetoacetyl-CoA (PDB:4NBU; Javidpour et al. 2013) indicates that Ser142 binds the C3 carbonyl oxygen of the acetoacetyl moiety, positioning the substrate adjacent to the nicotinamide portion of NADPH and the terminal hydroxyl group of Tyr155, which also forms hydrogen bonds with the C3 carbonyl oxygen. Catalysis is provided by the transfer of a hydride from the nicotinamide of NADPH to the C3 carbon of the substrate forming an alkoxide enolate transition state, which then accepts a proton from Tyr155 and resolves to form the β-hydroxyacyl-ACP product, resulting in the dissociation of the now oxidised co-factor from the binding pocket.

Importantly, the proton transfer from Tyr155 is replenished by a proton relay, in which a proton is transferred through a series of highly ordered water molecules and conserved residues. Within the NmFabG:NADPH complex, the proton is transferred from Thr14 and Asn89 through three water molecules to Asn110, then another water molecule to Lys159, and through the nicotinamide ribose hydroxyls of NADPH to Tyr155. This proton relay is abolished in absence of the co-factor in apo EcFabG, and thus likely induced by the rotation of Lys159 toward the nicotinamide ribose moiety upon co-factor binding, resulting in the formation, dissolution, and hence replenishment of the proton wire with every catalytic cycle (Price et al. 2001, Price et al. 2004, White et al. 2005).
5.2.4 *NmFabG* co-factor interactions and induced conformational changes

Each monomer of the FabG tetramer contains a single NADPH binding site that stretches between the C-terminal ends of β1 and β4 within the twisted β-sheet of the Rossmann fold, extending toward β7 and β8 (Figure 5.5). The adenine ring of the co-factor forms hydrogen bonds with residues Asn63 and Ser64 of the β3-α3 loop, while residues Ser17, located between β1 and α1, and Gly92, from the β4-α4 loop region, bind the adjacent ribose. The ribose phosphate is stabilised through hydrogen bonding with Ser17, Thr40, and Arg18. No contacts appear to be made with the adenine adjacent phosphate of the pyrophosphate moiety; however, the nicotinamide adjacent phosphate engages in hydrogen bonding interactions with Ile20 and Thr190. The nicotinamide ribose participates in hydrogen bonding with residues Tyr155 and Lys159, while the nicotinamide moiety itself is involved in hydrogen bonding with the flexible α6/α7 subdomain that forms the “lid” of the active site (Figure 5.5).

Comparison of the *EcFabG* and *EcFabG*NADP⁺ structures solved by Price et al. (2004) reveals several conformational changes are induced by co-factor binding. Upon binding of NADPH the region between *EcFabG* residues Thr135 and Ala152 (*NmFabG* Asn139-Ala156) shifts to accommodate the co-factor. Residues 143-152 are disordered in the apo *EcFabG* structure, however Val140 and Thr142 (*NmFabG* Val144 and Val146) are clearly present within the molecule and appear to shift ~6.5 Å and ~14.5 Å respectively in the NADPH bound structure, demonstrating a conformation change in this region. The active site serine (Ser138; *NmFabG* Ser142) shifts ~3.8 Å and rotates 180°, which appears to allow the side chain of Tyr151 (*NmFabG* Tyr155) to rotate roughly 120°, with the terminal hydroxyl group arranged adjacent to the nicotinamide ribose of the co-factor. The side chain of Lys155 (*NmFabG* Lys159) also bends 90° towards the
nicotinamide ribose, allowing the NZ nitrogen atom to form hydrogen bonds with the 2'- and 3'-hydroxyl groups of this moiety.

In the EcFabG:NADPH binary structure, residues 143-152, which were disordered in the apo EcFabG structure, form a helix turn helix structure which lies adjacent to the α5-α5' dimer interface, stabilised by hydrogen bonding between Ser138 and Gly141, and Asn93 and Gly147. Furthermore, residues Asn86-Met96 (NmFabG Asn90-Met100) shift up to 4.2 Å to avoid a steric clash with the nicotinamide and nicotinamide ribose, with the carboxyl group of Asn86 and the ca of Ala87 being flipped roughly 180°. Price et al. (2004) suggest this disrupts hydrophobic packing between Ile89 and Met188 (NmFabG Ile93 and Met192), and breaks hydrogen bonds between the amide of Arg91 (NmFabG Ile95) and the hydroxyl group of the Tyr151. This in turn rotates into the active conformation, allowing the loop region of Asn86-Met96 (NmFabG Asn90-Met100) to pack closer together and accommodate the co-factor. The new conformation of the loop results in salt bridge formation between Arg91 and Asp105, and hydrogen bonding between the amide nitrogen and carboxyl group of Ile89 and the side chain of Asn110. The confirmation is further supported by hydrophobic packing between Ile89 and Ile106, and Val60 and Ala87.

Conversely to the co-factor induced conformational changes observed in EcFabG (Price et al. 2004), comparison of the apo NmFabG and the NmFabG:NADPH structures reveal few conformation changes occurring upon co-factor binding. Residues Asn139-Ala156 (Thr135-Ala152 in E. coli) in apo NmFabG and the binary complex closely mimic that of the EcFabG:NADPH complex, with this region having formed an ordered loop, with Ser142 (EcFabG Ser132) adopting the same “active” conformation. The remaining residues of the active site catalytic triad also adopt the same conformation as their
EcFabG:NADPH counterparts in both the apo NmFabG and NmFabG:NADPH structures. Tyr155 and Lys159 are rotated toward the hydroxyl groups of the nicotinamide ribose, with Lys159 forming hydrogen bonds with the 2’ and 3’-hydroxyl groups of the nicotinamide ribose, while the terminal hydroxyl group of Tyr155 binds to the 2’-hydroxyl group of the nicotinamide ribose. The proton relay observed in EcFabG:NADPH is also present in both NmFabG structures. Of the residues involved in creating this water network, only Lys155 displays a significant conformational change upon co-factor binding, thus rotation of the Lys155 side chain is the likely key conformational change required for formation of this relay. Likewise, residues Asn90-Met100 of the NmFabG structure (EcFabG Asn86-Met96) adopt the shifted conformation observed in the EcFabG:NADPH complex, accommodating the nicotinamide half of the co-factor.

A similar lack of conformation change was observed in the structures of FabG from Plasmodium falciparum (PfFabG) (Wickramasinghe et al. 2006). The apo PfFabG structure contains a sulphate molecule located in the position occupied by the 2’ phosphate of the NADPH adenosine ribose, and makes equivalent interactions to that of this phosphate. In apo PfFabG, the sulphate is bound by the terminal guanidino group of Arg72, whereas the equivalent residue in EcFabG, Arg15, forms a salt bridge with Asp187. In the co-factor bound structure, the side chain of this residue rotates 180°, stabilising the negative charge of the phosphate. Apo NmFabG contains a phosphate ion, which forms hydrogen bonds with the terminal nitrogen atoms of Arg18, and like the equivalent residues in EcFabG and PfFabG, makes similar contacts to the adenine ribose 2’ phosphate of the co-factor. This would imply that the binding of the phosphate/sulphate ion induces the conformational changes required for co-factor
binding, and that the salt bridge between Arg15 and Asp187 (Arg19 and Asp191) in 
NmFabG plays a crucial role in the conformation of the active site.

Figure 5.5 - FabG:NADPH interactions and proposed reaction mechanism
(A) NADPH (yellow) bound to FabG binds in a hydrophobic pocket, as indicated by 
the intensity of blue coloured patches. (B) The catalytic mechanism of FabG proposed 
by Price et al. (2004), Ser142 and Tyr155 form hydrogen bonds to the ketone at C3 of 
the substrate. Tyr155 and Lys159 rotate towards the nicotinamide ribose of the 
co-factor to form hydrogen bonds with the 2' hydroxyl, and 2'- and 3'-hydroxyl groups 
of the ribose. NADPH donates a hydride to the C3 carbon of the substrate, and a 
proton is transferred from Tyr155. The proton from Tyr155 is replenished by a proton 
relay, in which a proton is transferred from Thr11 and Asn85 through three water 
molecules to Asn110, then another water molecule to Lys159, and through the 
nicotinamide ribose hydroxyls of NADPH to Tyr155. (C) A 2D Schematic 
representation of the interactions between FabG and NADPH, hydrogen bonds are 
represented by green dashed lines; hydrophobic contacts are shown as semi-circular 
arcs. Figure (B) adapted from Price et al. (2004).
This is supported by the structure of FabG from *Bacillus anthracis*. Like *NmFabG* and *PfFabG*, the binding pocket of this structure appears to be in an ordered conformation similar to that of co-factor bound *EcFabG*, despite the absence of co-factor or ions within the co-factor binding site. In *Bacillus anthracis* FabG, the salt bridge between Arg15 and Asp187 that is found in apo *EcFabG* is abolished by the mutation of the aspartic acid to threonine, with the arginine residues found in the same position as in the *EcFabG*:NADPH complex (Price et al. 2004, Zaccai et al. 2008). Fluorescence spectroscopy studies that show both *EcFabG* and *PfFabG* exhibit negative cooperative binding of NADPH to FabG, implying that these enzymes must undergo some conformational change upon co-factor binding. Wickramasinghe et al. (2006) suggest the binding of a sulphate ion to *PfFabG* mimics an intermediate open conformation. Based on comparison of *NmFabG*, *PfFabG* and *Bacillus anthracis* FabG structures to that of the *EcFabG*:NADPH complex, absence of the salt bridge between Arg15 and Asp187 within apo *NmFabG*, *PfFabG* and *Bacillus anthracis* FabG structures suggests that disruption of this salt bridge plays an important role in active site conformation, and that binding of a phosphate ion within the adenine ribose 2’ phosphate binding pocket induces similar conformational changes in *NmFabG* to the co-factor induced conformational changes observed in *EcFabG*. However, the structure of *NmFabG* without any molecules in the co-factor binding site would be needed to confirm this theory.

The structure of *NmFabG* in complex with NADPH shows strong and well defined density for the adenine and adenine ribose moieties, however density for the nicotinamide and adjacent ribose is poorly defined in some molecules. Weak electron density for this part of NADPH has been previously reported in a number of FabG crystal structures, including that of *EcFabG* and FabG1 (MabA) from *M. tuberculosis,*
which indicates this portion of the co-factor may be flexible, binding in a two stage mechanism (Price et al. 2004, Cohen-Gonsaud et al. 2005). Mutation of the *EcFabG*:NADPH active site tyrosine (*EcFabG* Tyr151, Tyr155 in *NmFabG*) to phenylalanine by Price et al. (2004) yielded a crystal structure in which only the nicotinamide and nicotinamide ribose proteins are not visible. Comparison between the *EcFabG* Tyr151Phe mutant to wild type *EcFabG* revealed the nicotinamide adjacent phosphate of the diphosphate moiety had shifted ~3.5 Å and rotated roughly 90°, and was interacting with Arg91 (*NmFabG* Arg95), a residue not involved in co-factor interactions within the wild type *EcFabG*:NADPH structure. The adenine, adenine ribose, and adenine ribose 2’ phosphate moieties, however, all share the same interactions and active conformation as the wild type complex. The tight binding of the adenine half of NADPH in these *EcFabG*:NADPH structures led Price and co-workers (2004) to suggest that co-factor binding occurs via a two stage mechanism, where the adenine portion of NADPH binds first and induces a conformational change allowing the nicotinamide portion to bind. This is consistent with the apo *NmFabG*, *PfFabG* and *Bacillus anthracis* FabG structures, where disruption of the salt bridge by either an ion within the adenine ribose 2’ phosphate binding pocket or mutation, appears to induce the active site conformation required for catalysis. Furthermore, these structures suggest that binding of the adenine ribose 2’ phosphate moiety is responsible for initiating the conformational changes observed between *EcFabG* and *EcFabG*:NADPH and inducing an active conformation.

**5.2.5 Putative *NmFabG* substrate and ACP binding interactions**

While FabG preferentially binds ACP linked β-ketoacyl thioesters, CoA linked substrates can be utilised, at least *in vitro*, due to the phosphopantetheine arm shared by holo-ACP and CoA, thus interactions between FabG and the phosphopantetheine arm of
ACP can be inferred through those interactions observed when bound to CoA linked substrates. The crystal structure of FabG from *Bacillus sp.* strain SG-1 in complex with NADPH and the CoA linked β-ketoacyl thioester, acetoacetyl-CoA (PDB:4NBU), and FabG4 from *Mycobacterium tuberculosis* in complex with NAD+ and hexanoyl-CoA (PDB:3V1U), reveal that the flexible “lid” formed by helices α6 and α7 shift closer to the substrate, with the side chain of a methionine residue (*NmFabG* equivalent Met192) aligned parallel to the substrate, positioned between the substrate and the diphosphate moiety of the co-factor. The oxygen atom of the C3 keto group of the substrate is positioned ~2.5 Å from the hydroxide group of the active site tyrosine and the terminal oxygen atom on the side chain of the active site serine, and ~3 Å from the pyridine ring within the nicotinamide portion of the co-factor, allowing the transfer of protons through the active site tyrosine and co-factor to the fatty acid intermediate (Figure 5.5). The oxygen of the C1 ketone is stabilised by hydrogen bonding to the side chain nitrogen of Gln155, which possibly aids in stabilising the formation of the negative enolate transition state during catalysis. The pantetheine arm of the CoA/ACP molecule is stabilised through hydrogen bonding between the terminal phosphate of the phosphopantetheine moiety and the terminal nitrogen of Lys104, the nitrogen (N4P) and oxygen (O5P) atoms adjacent to the pantetheine thiol, and the side chains of Asp99 and Asn152 respectively, to further stabilise the substrate during catalysis.

Like the condensing enzymes FabH and FabF, *EcFabG* possesses an electropositive and hydrophobic area adjacent to the active site. Two conserved arginine residues located at the entrance to the co-factor/substrate binding site of the opposing monomer, Arg129 and Arg172 (Arg133 and Arg176 in *NmFabG*), are crucial for ACP binding (Zhang et al. 2003). Mutation of these residues significantly reduced the binding of ACP to FabG without affecting the overall structure or altering the catalytic ability of the enzyme.
Furthermore, mutation of these arginine residues to negatively charged glutamate residues showed a greater disruption to binding, with the most pronounced effect observed in the Arg129Glu and Arg172Glu double mutant, which displayed a rate of catalytic reduction of β-ketobutyryl-ACP 50 fold lower than β-ketobutyryl-CoA, thus confirming the essentiality of these residues and the electropositive area near the active site in interactions with ACP. The requirement of positively charged residues or a positively charged region for ACP binding is not surprising, considering a large proportion of the ACP molecule is acidic or negative charged at physiological pH in most bacterial species (Byers et al. 2007).

Although there is no structure of FabG in complex with ACP available, modelling a holo-ACP molecule onto the acetoacetyl-CoA molecule from the structure of Bacillus sp. strain SG-1 FabG in complex with acetoacetyl-CoA, and the structure of apo-NmFabG, indicates interactions involving several conserved residues (Figure 5.6). This model indicates Arg127, Arg133, and Arg176 (NmFabG numbering) form electrostatic interactions with a number of glutamate and aspartate residues, including Glu47, Glu53, Asp56, Glu57 and Glu58, which are conserved amongst E. coli, S. aureus, S. pneumoniae, H influenza, and Neisseria spp. ACPs (Appendix 1). The position of Arg127, Arg133 and Arg176 near the active site of an adjacent monomer, yet distant from the active site of the monomer in which they reside, provides an insight into the requirement of FabG dimerisation for catalytic activity, as well as the mechanism of inhibitors known to disrupt FabG dimer interfaces.
5.2.6 Potential NmFabG inhibitors and interactions

A number of FabG inhibitors have been identified, including chlorogenic acid, cinnamic acid derivatives, allosteric inhibitors, and some green tea catechins such as EGCG.
(Zhang et al. 2004, Li et al. 2006a, Tasdemir et al. 2006, Kristan et al. 2009, Cukier et al. 2013). However, the mechanism by which these molecules inhibit FabG has been determined for only a few inhibitors.

Certain cinnamic acid derivatives have been shown to have inhibitory activity against FabG. While there is no data indicating the method of inhibition of the cinnamic acid derivatives reported by Kristan et al. (2009), these inhibitors share a similar chemical structure to chlorogenic acid (Figure 5.7), itself an ester of dihydroxy-cinnamic acid and quinic acid. Thus, it would appear likely that these inhibitors interact with FabG in a similar manner. Inhibition of FabG by chlorogenic acid occurs via a competitive mechanism with respect to NADPH. Li et al. (2006a) suggest chlorogenic acid occupies the co-factor binding site based on the competitive mechanism of inhibition and the structural similarity to the portion of NADPH containing the diphosphate and ribose moieties (Figure 5.7). This may appear likely, however, chlorogenic acid and related cinnamic inhibitors may bind at a site unrelated to the NADPH binding site, yet still induce a conformational change incompatible with co-factor binding.

Unfortunately, chlorogenic acid is a poor inhibitor of EcFabG with an IC₅₀ of 88.1 µM, and also displays similar competitive inhibition against the fatty acid synthase isolated from duck liver (Li et al. 2006a). Combined, these two characteristics occlude the use of chlorogenic acid as an effective antimicrobial treatment; however, as one of the more characterised inhibitors of FabG, it may serve as a basis for fragment-based drug discovery or structure-based drug design if the binding site and interactions can be confirmed.
Li et al. (2006a) also suggest that EGCG inhibits FabG through competition with the co-factor NADPH, due to structural similarities between these two molecules (Figure 5.7). EGCG has been demonstrated to inhibit both EcFabG and *Plasmodium falciparum* FabG (*Pf*FabG), however the mechanism of this inhibition is uncertain. Zhang et al. (2004) report that EGCG inhibits EcFabG in a competitive manner in regards NADPH, but also inhibits co-factor bound EcFabG, whereas Li et al. (2006b) suggest that EGCG inhibits FabG by covalently modifying FabG, causing the enzyme to aggregate. EGCG was confirmed to inhibit *Nm*FabG with an IC$_{50}$ value of $\approx 7$ µM through enzymatic assays performed as per section 2.2.16, which is similar to that reported by Zhang et al. (2004) (5 µM).

**Figure 5.7 - Inhibitors of FabG.** Many FabG inhibitors share a similar structure to that of the enzyme co-factor NADPH (A). Li et al. (2006a) suggest chlorogenic acid (B) and EGCG (C) bind to the co-factor binding site based on the competitive mechanism of inhibition and the structural similarity to the portion of NADPH (A). The structurally similar cinnamic acid derivative (D) identified by Kristan et al. (2009) and chalcones such as Isoliquiritigenin (E) may also bind in a similar manner.
To confirm the mechanism through which EGCG inhibits FabG, X-ray crystallography of *NmFabG* in complex with EGCG was attempted. Unfortunately, efforts to crystallise *NmFabG* in complex with EGCG were unsuccessful. Soaking apo *NmFabG* crystals with various concentrations of EGCG caused the protein crystals to rapidly dissolve. Co-crystallisation of *NmFabG* with EGCG at molar ratios of 1:5, 1:10, 1:20, and 1:50, in the conditions used to produce apo *NmFabG* crystals, and conditions determined using the commercial crystallography screens listed in section 2.2.17 were also attempted, and while *NmFabG* was crystallised in the presence of EGCG, none of the diffraction quality crystals obtained contained bound ligand. The inability to achieve crystals of EGCG in complex with *NmFabG* may be due to the limited stability of EGCG, which is reported to degrade rapidly in aqueous solutions. The stability of EGCG can be enhanced by low temperature, low pH, and the presence of reducing agents (Dube et al. 2010), however co-crystallisation experiments using TCEP and/or conducted at 4 °C failed to produce any crystals. These issues may be overcome by further optimisation of pH and temperature. Alternatively, the creation of EGCG derivatives with greater stability may allow crystallisation of EGCG in complex with *NmFabG*. In the interim, superposition of *NmFabG* and the structure of *NmFabI* (detailed in chapter 5.3) indicates EGCG could competitively inhibit NADPH by binding at a site that overlaps with the binding site for the adenine moiety of NADPH, forming hydrogen bonds with Asn63 and Asn109 (Figure 5.8), yet these interactions need to be confirmed experimentally to determine if EGCG does bind at this location.
High throughput enzymatic drug screening by Cukier et al. (2013) identified several inhibitors that bind within the same hydrophobic cavity located at the A/D dimer interface. One of the most potent inhibitors identified, 2-phenyl-4-(1,2,4-triazol-4-yl) quinazoline (FG01), was found to inhibit *Pseudomonas aeruginosa* FabG (PaFabG) in a non-competitive mechanism with respect to NADPH, with an \( IC_{50} \) value of 0.02 \( \mu \text{M} \) (Cukier et al. 2013). Binding of FG01 to the FabG dimer interface was found to have little effect on the quaternary structure of the enzyme, yet significant conformational changes are present compared to the apo, and NADPH bound PaFabG structures. In the PaFabG:FG01 complex, helices \( \alpha 4 \) (residues 103–124) and \( \alpha 5 \) (residues 152–166), loops \( \beta 4–\alpha 4 \) (residues 90–102) and \( \beta 5–\alpha 5 \) (residues 140–151) which connect the N-termini of helices \( \alpha 4 \) and \( \alpha 5 \) to the core of the protein, as well as the four C-terminal residues of the monomer (residues 244–247) appear to have shifted, resulting in a cavity to accommodate the inhibitor. Importantly, residues 138–142 clash with the

**Figure 5.8 - Possible interactions between FabG and epigallocatechin gallate.**

Superposition of the structures of *NmFabG* (cyan), and *NmFabI* (silver) in complex with EGCG (yellow) reveals little deviation in the secondary structure of the two enzymes (A) and a number of possible hydrogen bonds (yellow dashed lines) which may form between EGCG and FabG within in the adenine portion of the co-factor binding site (B).
nicotinamide ring of NADPH, resulting in the active site tyrosine and lysine residues being rotated away from the 2’- and 3’-hydroxyl groups of the co-factor nicotinamide ribose, much like that in apo EcFabG, thus the complex is incompatible with co-factor binding (Cukier et al. 2013).

Superposition of the crystal structures of apo PaFabG and PaFabG:FG01 reveals a stark contrast between the 138-146 binding loop. In PaFabG and PaFabG:NADPH, the 138-146 binding loop forms a helix turn structure which packs tightly against helix 149-154, presumably to allow co-factor binding as it does in the EcFabG:NADPH complex. Binding of FG01 however, causes this loop to bulge into the co-factor binding domain and clash with the nicotinamide ring of NADPH. These conformational changes appear to be opposite to those observed upon NADPH binding to EcFabG, thus it is uncertain whether FG01 prevents the conformational changes induced by the adenine portion of NADPH required for co-factor binding, or induces conformational changes incompatible with NADPH binding itself. Regardless, FG01 appears to be a potent inhibitor; however, the efficacy of this inhibitor in vivo and its clinical spectrum are yet to be determined. Whilst not as potent as FG01, EGCG possesses the distinct advantage of being able to inhibit both FabG and FabI, however further modification is required to enhance the pharmacological and antimicrobial properties to levels suitable for therapeutic use.
5.3 *Neisseria* FabI

5.3.1 Structure determination

The gene encoding the enoyl-ACP reductase of *N. meningitidis* FabI (*Nm*FabI; accession no. YP_975773.1) was amplified from genomic DNA and cloned into the expression vector pMCSG21 via ligation independent cloning (LIC) as described in sections 2.2.5-2.2.10 and previous chapters. Colony PCR (section 2.2.11) was used to screen spectinomycin resistant colonies, purified plasmid DNA was isolated from colonies containing recombinant *Nm*FabI as per section 2.2.3, and sequenced (section 2.2.12) to ensure the fidelity of the clone. Recombinant *Nm*FabI was expressed using auto-induction media and purified by Ni$^{2+}$ affinity and size exclusion chromatography as per sections 2.2.13 and 2.2.14. Purified *Nm*FabI was concentrated to ~60 mg/mL via Amicon centrifugal device, and was assessed to be ~95% pure by SDS-PAGE (section 2.2.16).

Initial sparse matrix crystallisation trials were performed using undiluted *Nm*FabI as per section 2.2.17. Small tetrahedral shaped crystals were observed in condition No. 32 of Hampton Research crystal screen (2 M ammonium sulphate) following overnight incubation. Single large tetrahedral shaped crystals were obtained in 2 M ammonium sulphate after a period of ~4 days using a 1:2 dilution of recombinant *Nm*FabI in S200 buffer (Table 2.6). Prior to collection of X-ray diffraction data, crystals were cryoprotected in 25% glycerol and flash cooled in liquid nitrogen. X-ray diffraction data were collected at a wavelength of 0.9537 Å on the Australian Synchrotron MX2 microcrystallography beamline. A total of 200° of rotation were collected at 0.5° oscillations. Data were integrated and scaled using iMosflm and Aimless to a resolution of 2.15 Å.
The large tetrahedral crystals displayed $P6_{1}22$ symmetry with the unit cell parameters $a = 91.61$, $b = 91.61$, $c = 241.5$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$.

Two $NmFabI$ molecules were estimated in the asymmetric unit, resulting in a solvent content of 53.78% and a Matthews coefficient of 2.66 Å$^3$/Da. This was confirmed by molecular replacement using a monomer of FabI from *Burkholderia pseudomallei* (PDB:3EK2; Baugh et al., 2013) as the search model and an estimated 2 molecules within the asymmetric unit. Following model building and structure refinement as described in section 2.2.18, the final model contained no Ramachandran outliers, 0.3% rotamer outliers, and displayed final R and R-free values of 0.178 and 0.203 respectively (for further details see Table 5.2).

Crystals of $NmFabI$:NAD$^+$ complex were obtained by co-crystallisation of recombinant $NmFabI$ with NADH at a molar ratio of 1:10 respectively, and screened against a range of ammonium sulphate concentrations as per apo $NmFabI$. Additionally, in an effort to reduce nucleation and yield larger crystals, crystallisation was also performed with glycerol concentrations ranging from 5 – 12.5%. A single large crystal displaying similar morphology to those of apo $NmFabI$ was obtained in 2.3 M ammonium sulphate and 12.5% glycerol. Prior to collection of X-ray diffraction data, crystals were cryoprotected in 25% glycerol and flash cooled in liquid nitrogen as per apo $NmFabI$.

One hundred and eighty degrees of X-ray diffraction data were collected on the Australian Synchrotron MX2 micro crystallography beamline at 0.5° oscillations. Data were processed as per apo $NmFabI$ and scaled to a resolution of 2.25 Å. The $NmFabI$:NAD$^+$ crystal displayed the same symmetry as those of apo $NmFabI$, and similar unit cell lengths ($a = 91.10$, $b = 91.10$, $c = 240.84$ Å). As such, two $NmFabI$
molecules were expected in the asymmetric unit, as found in the crystal structure of apo
NmFabI. This was confirmed by molecular replacement using a monomer of apo
NmFabI as a search model. NAD$^+$ molecules were modelled as per the
NmFabG:NADPH structure. Briefly, NAD$^+$ molecules were placed by a combination of
Phenix ligand fit, superposition of EcFabI in complex with NAD$^+$ (PDB:1DFI) and the
NCS ligands function in WinCoot. NAD$^+$ molecules were then further modelled in
WinCoot. A total of two NAD$^+$ molecules were observed in the asymmetric unit. The
final NmFabI:NAD$^+$ model displayed R and R-free values of 0.170 and 0.189
respectively. Other model and data collection statistics are listed in Table 5.2.

NmFabI:NAD$^+$:triclosan crystals were obtained by co-crystallisation as per the
NmFabI:NAD$^+$ crystal, using a 1:2.5:5 molar ratio of recombinant NmFabI to NADH to
triclosan. Crystals grown in 1.7 M ammonium sulphate were selected for data
collection, cryoprotected in 25% glycerol and flash cooled in liquid nitrogen as per
other NmFabI crystals. X-ray diffraction data were collected on the Australian
Synchrotron MX2 micro crystallography beamline at 0.5° oscillations for 180° of
rotation, as per the NmFabI:NAD$^+$ complex. Diffraction data were then integrated in
iMosflm, and merged and scaled to a resolution of 1.7 Å using Aimless. The crystal
displayed P6$_1$22 symmetry as per the previous NmFabI crystals, and was confirmed to
have two molecules of NmFabI in the asymmetric unit by molecular replacement using
apo NmFabI as the search model. NAD$^+$ molecules were placed by superposition of the
NmFabI:NAD$^+$ model, and triclosan molecules were placed using Phenix ligand fit.
Following model building and refinement, the final model displayed an R-value of
0.168 and R-free of 0.186.
Co-crystallisation of *Nm*FabI and EGCG using the same conditions as apo *Nm*FabI and *Nm*FabI complexes failed to yield any crystals. Attempts to obtain *Nm*FabI:EGCG crystals by soaking the *Nm*FabI crystals overnight with various concentrations of EGCG also failed to provide diffraction quality crystals, with the apo *Nm*FabI crystals dissolving upon prolonged contact with EGCG. As such, *Nm*FabI:EGCG crystals were obtained by soaking apo *Nm*FabI crystals in cryoprotectant containing 1.75 M ammonium sulphate, 25% glycerol and 100 mM EGCG for ~1 minute, before flash cooling in liquid nitrogen. A total of 180° of X-ray data were collected at 0.5° oscillations on the Australian Synchrotron MX2 micro crystallography beamline, as per other *Nm*FabI structures. Data were integrated, processed, and scaled to 2.20 Å, as per section 2.2.18. The crystal displayed P65 symmetry and similar unit cell parameters to that of other *Nm*FabI structures, indicating the presence of 2 *Nm*FabI molecules in the asymmetric unit. This was confirmed by placement of the apo *Nm*FabI dimer into electron density by rigid body refinement. EGCG molecules were then generated in Phenix elbow, and placed using Phenix ligand fit. Two EGCG molecules were located within the asymmetric unit, and modelled in WinCoot. Model building and refinement was performed using WinCoot and Phenix refine respectively. Data collection and final model statistics for the *Nm*FabI:EGCG complex are provided in Table 5.2.
Table 5.2 - NmFabI diffraction data and model statistics.

<table>
<thead>
<tr>
<th></th>
<th>apo-NmFabI</th>
<th>NmFabI:NAD</th>
<th>NmFabI:NAD:Triclosan</th>
<th>NmFabI:EGCG</th>
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<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>31.64-2.15 (2.23-2.15)</td>
<td>35.40-2.25 (2.24-2.20)</td>
<td>34.47-1.90 (1.94-1.90)</td>
<td>47.97-2.20 (2.27-2.20)</td>
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<tr>
<td>Space group</td>
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<td>P6_122</td>
<td>P6_122</td>
<td>P6_122</td>
</tr>
<tr>
<td>Unit cell length (Å)</td>
<td>a = 91.93, b = 91.93, c = 241.71</td>
<td>a = 91.10, b = 91.10, c = 240.84</td>
<td>a = 90.77, b = 90.77, c = 241.27</td>
<td>a = 91.48, b = 91.48, c = 241.07</td>
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<tr>
<td>Unit cell angle (°)</td>
<td>α = 90, β = 90, γ = 120</td>
<td>α = 90, β = 90, γ = 120</td>
<td>α = 90, β = 90, γ = 120</td>
<td>α = 90, β = 90, γ = 120</td>
</tr>
<tr>
<td>Total observations</td>
<td>483234 (48782)</td>
<td>215086 (21505)</td>
<td>939035 (63952)</td>
<td>517397 (13845)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>31734 (3222)</td>
<td>29052 (2990)</td>
<td>47336 (3112)</td>
<td>31301 (2605)</td>
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<tr>
<td>Multiplicity</td>
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<td>7.4 (7.2)</td>
<td>19.8 (20.6)</td>
<td>16.5 (5.3)</td>
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<tr>
<td>Completeness (%)</td>
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<td>99.9 (100.0)</td>
<td>100.0 (100.0)</td>
<td>100.0 (99.5)</td>
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<tr>
<td>Mean I/sigma (I)</td>
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<td>13.9 (9.0)</td>
<td>21.6 (10.2)</td>
<td>16.5 (5.8)</td>
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<tr>
<td>CC (1/2)</td>
<td>0.997 (0.982)</td>
<td>0.988 (0.902)</td>
<td>0.998 (0.987)</td>
<td>0.988 (0.887)</td>
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<tr>
<td>R-pim</td>
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<td>0.024 (0.065)</td>
<td>0.033 (0.186)</td>
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<td>R-meas</td>
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<td>0.168 (0.442)</td>
<td>0.105 (0.295)</td>
<td>0.137 (0.419)</td>
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<td>R-merge</td>
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<td>0.169</td>
<td>0.208</td>
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<tr>
<td>R-free</td>
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<td>RMSD bonds (Å)</td>
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<td>0.007</td>
<td>0.005</td>
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<td>RMSD angles (°)</td>
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<td>0.970</td>
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<td>Ramachandran allowed (%)</td>
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<td>Ramachandran outliers (%)</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note: Highest resolution shell statistics are shown in parentheses; number of atoms does not include hydrogen atoms.
5.3.2 Overall structure of *NmFabI* and *NmFabI*:complexes

The asymmetric unit of the *NmFabI* crystal structures contain two monomers of ~27.5 kDa arranged as a homo-dimer, forming a tetramer with crystal symmetry. Like *NmFabG*, each *NmFabI* monomer contains a Rossmann fold nucleotide binding motif that is comprised of a seven stranded parallel β-sheet flanked by seven α-helices, and three small helical turns (Figure 5.9). The four monomers of the *NmFabI* homo-tetramer are related by D2 symmetry, forming three different interfaces between molecules A and B (A/B interface), molecules A and D (A/D interface), and molecules A and C (A/C interface). The most extensive interface is the A/C interface, which is formed between one molecule found in the asymmetric unit, and another generated via symmetry, and buries ~13% (~1670 Å) of the total surface area. This interface is mediated by a network of hydrogen bonding between α3, α4, α5, a helix turn helix structure (HTH) between β4 and α4, and the equivalent structures on the opposing monomer. The majority of the interactions involve α4, which forms hydrogen bonds with the loop joining β3 and α3, the HTH, α5 and its symmetry mate, while α5 also forms contacts with the HTH region and the loop connecting β5 and α5. The interface is also stabilised by a number of salt bridges between Asp68 of α3 and Arg110 of α4’, residues Asp103 and Asp106 of the HTH structure, and Arg132 of α4’, and between Glu118 of α4 and Arg110 on the opposing monomer.
Figure 5.9 - Tertiary and secondary structure elements of *N. meningitidis* FabI.

*NmFabI* forms a homo-tetramer through two sets of identical dimer interfaces in a similar manner to that observed in the *NmFabG* structure. One interface is formed through interactions between α3, α4, α5, a helix turn helix structure, and the equivalent motifs on the opposing monomer. The other interface is formed through the 7 stranded β-sheets of two monomers, that align parallel to that of the opposing monomer, forming a 14 stranded β-sheet almost identical to that present in *NmFabG* (A). The secondary structure elements of *NmFabI* at 1° (B), 90° (C) and 180° (D) rotation. A 2D schematic of the *NmFabI* topology (E) and protein sequence of *NmFabI* (F). α-helices, β-strands, and loops are coloured red, yellow, and green, respectively.
The A/B interface observed within the asymmetric unit is largely comprised of hydrophobic packing between helix α7 of each molecule, and strand β7, which arrange anti-parallel to β7 of the opposing monomer and forms hydrogen bonds between the carboxyl group of Gly240 and the peptide nitrogen of Asp249’ and Gly250’, and the side chain of Glu244 and the peptide nitrogen of Tyr247’. Additional contacts are made through the residues between α6 and α7, including interactions between Pro216 and Lys180’ at the C-terminus of α5’, and Arg219 and Glu225 with Ser239’ of α7’ in the opposing monomer. The A/C interface, a small interface concealing only 6.5% of the total surface area, is comprised of hydrogen bonding between Ala153 on the loop connecting strand β3 and helix α3 of molecule A, and Ile254 and Ala256 at the C-terminus of molecule C.

Since a probable tetrameric quaternary structure of NmFabI was observed to form through crystallographic symmetry, the quaternary structure of NmFabI was compared with the oligomeric state of other FabI proteins. Like FabG, the biological unit of FabI enzymes appears to vary with species. *Brassica napus* FabI (*Bn*FabI) (Rafferty et al. 1995), *E. coli* FabI (*Ec*FabI) (Baldock et al. 1996), and *B. subtilis* FabI (* Bs*FabI) (Kim et al. 2011) have been reported as tetramers in solution, whilst Schiebel et al. (2012) and Kim et al. (2010) suggest *S. aureus* FabI (*Sa*FabI) and *Bacillus cereus* FabI are dimeric in solution, yet tetramerise upon binding of co-factor and substrate, but not co-factor alone. Schiebel et al. (2012) further report that tetramerisation is also pH dependent, with *Sa*FabI forming a dimer at pH 8.0, yet a tetramer at pH 5.6. This is inconsistent with *Bn*FabI and *Ec*FabI, which were found to be tetrameric in the presence of NAD+, and in the case of *Bn*FabI, tetrameric at pH 8.0 (Rafferty et al. 1995, Baldock et al. 1996). Due to these varying reports, a combination of small angle X-ray scattering (SAXS) and gel filtration was used to determine the biological state of *Nm*FabI in
solution. Elution from the gel filtration revealed a native molecular weight of approximately 100 kDa, corresponding to a tetramer. This was further supported by SAXS measurements taken using 0.1, 0.25, 0.5, 1, 2.5, and 5 mg/mL of FabI. Superposition theoretical scattering curves generated using monomeric, dimeric and tetrameric assemblies onto the experimental scattering curves suggest NmFabI forms a tetramer when in a solution of 125 mM NaCl and 50 mM Tris pH 8.0, hence tetramerisation of NmFabI is not dependent upon co-factor binding, substrate binding, or low pH (Figure 5.10).

Figure 5.10 - Small angle X-ray scattering profiles of NmFabI. Theoretical scattering curves (blue lines) of an NmFabI monomer (A), dimer – A/B interface (B), A/D interface (C), A/C interface (D), and tetramer (E) superimposed over experimental NmFabI SAXS data (red dots) using CRYSOl show NmFabI forms a tetramer in solution at pH 8.0.
5.3.3 Putative active site and reaction mechanism of \textit{NmFabI}

As with other FASII enzymes the active site and catalytic mechanism has been well characterised in \textit{E. coli}. \textit{FabI} is an NADH dependent oxidoreductase of the short-chain dehydrogenases/reductases (SDRs) family of enzymes, which catalyses the reduction of the trans-2-acyl-ACP molecule to an acyl-ACP. SDRs characteristically possess a conserved Ser-Tyr-Lys catalytic triad, arranged in a Ser-Xaa10-Tyr-Xaa4-Lys motif.

\textit{FabI} is atypical in the sense that it contains a Tyr-Xaa6-Lys motif, in which the active site tyrosine and lysine are located 6 residues apart, and an active site serine, which usually stabilises the substrate of SDRs is absent (Rafferty et al. 1995, Baldock et al. 1996, White et al. 2005). The catalytic reduction of substrate involves the transfer of a hydride from the nicotinamide ring of the co-factor to the C3 carbon and C2–C3 double bond of the substrate (Figure 5.10). The development of an enolate anion on the C1 carbonyl oxygen is supported by the positive charge of the active site lysine and Tyr157, which donates a proton from the tyrosine hydroxyl to form an enol. The resulting enol then undergoes tautomerisation to form an acyl-ACP molecule.

\textbf{Figure 5.11 - Proposed \textit{FabI} reduction mechanism.} The catalytic mechanism of \textit{FabI} proposed by White et al. (2005). The catalytic reduction of the enoyl substrate is initiated by the transfer of a hydride from the nicotinamide ring of the co-factor to the C3 carbon and C2–C3 double bond of the substrate. NADH donates a hydride to the C3 atom of the enoyl substrate. Tyr157 then donates a proton from the side chain hydroxyl to the resulting enolate anion on the C1 carbonyl oxygen, forming an enol. The enol then undergoes tautomerization to form an acyl-ACP molecule. Figure adapted from White et al. (2005).
The co-factor binds within the active site pocket which stretches across loop regions adjacent to the C-terminal ends of β-strands β1, β2, β3, β4, β5 and β6 (Figure 5.11). The adenine ring of NADH is stabilised by hydrogen bonding between the N1 and N6 nitrogen atoms of the adenine ring, Val65, and Asp64, respectively. The 3’ hydroxyl of the adenine ribose forms a hydrogen bond with Gly13, while the diphosphate moiety forms hydrogen bonds with Ser19, Ile20, Thr195, and Ala197. Thr195 also interacts with the nicotinamide moiety, which is further stabilised by hydrogen bonds between the carboxyl and amide groups of Ile193, and the terminal nitrogen and oxygen atoms of the co-factor nicotinamide (Rafferty et al. 1995, Baldock et al. 1996, White et al. 2005) (Figure 5.11). Two crucial hydrogen bonds are formed between Lys164 and the nicotinamide ribose hydroxyls, linking the co-factor to a network of ordered water molecules, and forming a proton relay with Ser120, similar to that observed in NmFabG. This serves to replenish the proton donated by the active site tyrosine (White et al. 2005) (Figure 5.11). White et al. (2005) suggest this occurs through direct contact with the 2’ hydroxyl of the nicotinamide ribose of the co-factor, however inspection of NmFabI in complex with NAD⁺ and the crystal structures of FabI from Anaplasma phagocytophilum (PDB:3K31), B. napus (PDB:1ENP; 1ENO), Candidatus liberbacter (4NK5), and P. falciparum (PDB:1V35) reveals a water molecule between the 2’-hydroxyl of the nicotinamide ribose of the co-factor and the phenolic oxygen of Tyr157 in some chains. Furthermore, the distance between the ribose hydroxyl and the terminal oxygen of the active site tyrosine is 4 – 4.4 Å, which is too large a distance for favourable hydrogen bonding, thus the active site tyrosine may be linked to the co-factor by a water mediated hydrogen bond.
5.3.4 Putative *NmFabI* ACP and substrate binding interactions

Binding interactions between FabI and its substrate trans-2 enoyl-ACP, have been studied in *EcFabI* through a combination of X-ray crystallography, mutagenesis, and molecular dynamic simulations. Efforts to crystallise the acyl carrier protein from *N. meningitidis* in complex with FabI did not produce protein crystals, and so interactions between ACP and *NmFabI* must be conferred through studies performed with other FabI proteins. As with other Fab proteins, the interaction between FabI and ACP occurs through hydrophobic and electropositive residues on the surface of FabI, adjacent to a hydrophobic cavity leading to the active site, and hydrogen bonds that stabilise the phosphopantetheine arm and fatty acid intermediate.

Rafi et al. (2006) determined the crystal structure of ACP bound to FabI, however the active site serine of ACP, linked to the phosphopantetheine moiety, is positioned ~40 Å from the co-factor binding site, making delivery of the substrate to the active site residues impossible. Rafi et al. (2006) also determined residues Lys201, Arg204, and Lys205 located on the substrate binding loop (SBL) were crucial for the catalytic reduction of ACP-linked trans-2-dodecenoyl, but not trans-2-dodecenoyl-CoA. Of these three residues, only Lys205 (*NmFabI* Lys206) is present in *NmFabI*, however *NmFabI* does contain a number of basic residues positioned at the protein surface surrounding the co-factor binding site, including lysine residues 43, 56, 97, 194, and 205, and arginine residues 18, 47, and 62. The mechanism through which ACP binds FabI, as described by Rafi et al. (2006), is based upon interactions between FabI residues Lys201, Arg204 and Lys205, and ACP residues Asp35, Asp38, Glu41, and Glu48, and not the basic residues present in *NmFabI*. As such, it is uncertain how this mechanism applies to *NmFabI* and its interactions with ACP.
5.3.5 Conformational changes of *Nm*FabI binary and ternary complexes

Unlike FabG, the catalytic triad of FabI does not appear to undergo conformational changes upon co-factor binding, with an RMSD of 0.27 Å between apo *Nm*FabI and the *Nm*FabI:NAD$^+$ complex. However, a large conformational change occurs in the substrate binding loop (residues 190-205). In the apo structure, this loop is largely disordered, yet upon co-factor binding, Ile193, Thr195, and Ala197 of the loop are stabilised by hydrogen bonds to the pyrophosphate of the co-factor. While the full extent of the conformational change is unknown due to the disorder of this loop in the apo structure, residues Phe204 and Gly205 shift ~1.9 and 1.7 Å, respectively. The SBL in the *Nm*FabI:NAD$^+$ complex is ordered in one chain and partially ordered in the other, yet this region is fully ordered in the structures of *Nm*FabI bound by triclosan and NAD$^+$. Triclosan, a well characterised and widely used antibacterial agent, forms an adduct with NAD$^+$ and FabI, preventing binding of the reduced co-factor (NADH) which is required for catalysis. This appears to be mediated by hydrophobic packing between the substrate binding loop and helices α5 and α7, as the only bonds formed by triclosan involve the co-factor and active site lysine and tyrosine. The O17 atom of triclosan displaces the ordered water molecule that links to Try157 of the co-factor in the *Nm*FabI:NAD$^+$ structure. The nicotinamide ribose hydroxyls form the same hydrogen bonds to the active site lysine, with additional hydrogen bonds between the 2’ ribose hydroxyl and oxygen atoms O7 and O17 of the inhibitor.
Figure 5.12 - *N. meningitidis* FabI co-factor and inhibitor interactions. (A) NAD\(^+\) (yellow) bound to *Nm*FabI. The co-factor binds in a hydrophobic pocket (as indicated by blue surface) that stretches across the loop regions adjacent to the C-terminal ends of β-strands β1, β2, β3, β4, β5, and β6. The inhibitor triclosan (grey) forms an adduct with NAD\(^+\), preventing the oxidised co-factor from leaving the active site and thus preventing the reaction. (B) Superposition of the triclosan related FabI inhibitors CG400549 (pink; PDB:4CV2), AFN-1252 (cyan; PDB:4FS3), and a molecule with a backbone similar to that of MUT056399 (orange;PDB:3LT1), onto the *Nm*FabI:triclosan complex. (C) A 2D representation of the interactions between *Nm*FabI and NAD\(^+\) (purple), and (D) *Nm*FabI, NAD\(^+\) (brown), and triclosan (purple). Hydrogen bonds are represented by green dashed lines; hydrophobic contacts are shown as semi-circular arcs.
5.3.6 *Nm*FabI interactions with triclosan and related inhibitors

Although the bonds formed between triclosan and FabI involve only the active site lysine and tyrosine, the residues forming the small hydrophobic pocket in which triclosan binds are vital to its inhibition of FabI enzymes, with a number of point mutations affecting this area being shown to facilitate bacterial resistance to triclosan. Mutations in *Ec*FabI of Gly93Val in the loop region connecting β4 and α4, Met159Thr on helix α5, and Phe203Leu on helix α7 increase the MIC of triclosan by 95, 12 and 6 fold respectively compared to that of wild type *Ec*FabI, either due to disruption of the hydrophobic interactions between the enzyme and inhibitor, or steric clashes which are incompatible with triclosan binding (McMurry et al. 1998, Stewart et al. 1999).

Triclosan has been implicated in disruption of the endocrine system through binding of androgenic and oestrogenic receptors, and decreasing thyroid hormone levels. Triclosan has also demonstrated a carcinogenic property, stimulating the growth of both breast and ovarian cancer cell lines *in vitro* through an oestrogen receptor-dependent mechanism. However whether the level of environmental exposure to triclosan is high enough to induce these effects is still debated (Gee et al. 2008, Christen et al. 2010, Stoker et al. 2010, Witorsch 2014). In light of these potential adverse effects, the wide spread use of triclosan in products such as toothpaste, mouthwash, antibacterial creams, antibacterial soaps, and cleaning products, as well as its detection in soil and water, has aroused much debate. The wide spread use of triclosan has also been linked to an increase in triclosan resistant bacteria, particularly in *S. aureus* clinical isolates, causing further concern over its use in common household and personal care products (Suller et al. 2000, Yazdankhah et al. 2006).
The identification of FabI as the primary target of triclosan, as well as clinical isolates of methicillin resistant *S. aureus* (MRSA) resistant to triclosan, led to the development of FabI inhibitors targeted against *Sa*FabI. At least three of these inhibitors, AFN-1252, CG400549, and MUT056399, have entered into clinical trials and exhibit strong antibacterial activity. AFN-1252 is the most potent of these inhibitors, with an MIC$_{90}$ ranging from 0.008 - 0.015 µg/mL against various strains of MRSA (Karlowsky et al. 2009, Kaplan et al. 2012). This is followed by MUT056399 and CG400549, with MIC$_{90}$ values ranging from 0.03 - 0.12 µg/mL and 0.125 - 0.5 µg/mL respectively, however MICs against *N. meningitidis* and *N. gonorrhoeae* have only been published for MUT056399 with an MIC$_{90}$ of 0.25 µg/mL against *N. meningitidis* and *N. gonorrhoeae* (Park et al. 2007, Escaich et al. 2011). Superposition of the crystal structures of *Sa*FabI in complex with these inhibitors onto the structure of *Nm*FabI in complex with NAD and Triclosan reveal AFN-1252, CG400549, and MUT056399 all bind in the same hydrophobic pocket as triclosan (Figure 5.12), forming aromatic stacking interactions with the co-factor and hydrogen bonding with the active site tyrosine. As such, it is unsurprising that single point mutations similar to those which confer resistance to triclosan have been found to confer resistance to AFN-1252, MUT056399, and CG400549, with the mutations Ala95Val and Phe204Ser shown to confer resistance to MUT056399 (Escaich et al. 2011), Phe204Leu conferring resistance to CG400549 (Park et al. 2007), and Met99Thr conferring resistance to AFN-1252 (Yao et al. 2013). Superposition of AFN-1252, CG400549, and MUT056399 onto the structure of *Nm*FabI in complex with NAD$^+$ and Triclosan also indicate there are no steric clashes or mutations that would otherwise reduce the effectiveness of these compounds against *N. meningitidis* or *N. gonorrhoeae* (Figure 5.12). However, that resistance to triclosan, AFN-1252, MUT056399, and CG400549 can be conferred through single point mutations within the same hydrophobic binding pocket indicates a clear limitation in
these inhibitors and other inhibitors, which may interact with FabI in the same binding site. It has been suggested that the incidence of resistance conferred through a single genetic change can be reduced by simultaneously inhibiting multiple targets (Silver 2011). In this context, epigallocatechin gallate, which inhibits both FabG and FabI, and appears a promising lead compound for the development of such antimicrobials.

5.3.7 Inhibition of NmFabI by epigallocatechin gallate

Epigallocatechin gallate (EGCG) and related green tea catechins have been shown to inhibit EcFabI in a competitive manner in regards to the co-factor NADH, and thus must bind in a manner different to triclosan and related inhibitors, which share the same binding site. EGCG was shown to inhibit EcFabI with an IC$_{50}$ value of 15 μM, yet exhibited poor antimicrobial activity in E. coli that contained a mutation in the TolC efflux pump, indicating EGCG is rapidly deactivated or exported in E. coli, and may be less effective in wild type E. coli that possess a functional efflux system. However, N. gonorrhoeae and N. meningitidis do not possess this innate resistance to EGCG. As demonstrated by Matsumoto et al. (2012), EGCG exhibits an MIC of 32 μg/ml, while an acylated derivative comprised of EGCG attached to a 16 carbon fatty acid (palmitate) displayed greater antimicrobial activity, with an MIC of 16 μg/ml. Activity assays, performed as per section 2.2.16, showed EGCG also inhibited NmFabI in a concentration dependent manner with an apparent IC$_{50}$ of 17 μM.

To determine the manner of this inhibition and the mechanism through which EGCG binds to NmFabI, the structure of NmFabI in complex with EGCG was solved by X-ray crystallography. EGCG partially occupies the co-factor binding site, with the O07 of the dihydroxy benzopyran moiety of the inhibitor potentially forming hydrogen bonds with the peptide nitrogen of Val65 and the carbonyl oxygen of Cys63, and oxygen
atoms O1 of the trihydroxyphenyl group and O44 of the gallate moiety forming hydrogen bonds with Asp64. These hydrogen bonds are further supported by extensive hydrophobic packing interactions involving Val40, Val41, Ala66, Ile92, Gly93, Phe94, and Ile119 (Figure 5.13).

The site at which EGCG is bound to NmFabI is similar to that observed during docking of EGCG to InhA (Mycobacterium tuberculosis FabI) by Sharma et al. (2008), however the conformation differs from that reported. Sharma et al. (2008) reported that the gallate moiety of EGCG forms T-shaped aromatic interactions with the side chain of the Phe97, the equivalent of Phe94 in NmFabI. The gallate moiety of EGCG does arrange in a T-shaped orientation to Phe94 in the structure of NmFabI bound to EGCG, yet at a distance of ~8 Å apart, thus aromatic interactions between these molecules appears unlikely. Weak parallel displaced aromatic stacking interactions may occur between Phe94 and the benzopyran moiety of EGCG, with NmFabI aligning almost parallel to the aromatic ring of the Phe94 side chain at a more favourable distance of 4.5 Å.

As a therapeutic agent, epigallocatechin gallate is relatively unsuitable due to potential interactions with other human proteins shown to bind EGCG, such as cardiac troponin C (Robertson et al. 2009), peptidyl prolyl cis/trans isomerase (Pin1) (Urusova et al. 2011), and the thyroxine transporting protein transthyretin (Miyata et al. 2010). Furthermore, the antimicrobial activity exhibited by EGCG and acylated derivatives are comparatively weak when compared to that of AFN-1252, MUT056399, or CG400549. Yet as a lead compound, EGCG possesses two distinct advantages. Firstly, EGCG occupies a portion of the co-factor binding site and forms hydrophobic interactions and hydrogen bonds similar to those formed with NAD. As such, mutations conferring resistance to EGCG are likely unfavourable and could disrupt co-factor binding and
potentially reduce catalytic activity. Secondly, EGCG has been shown to inhibit FabG and FabI, and due to the similarity of these enzymes, particularly in regards to their active sites, the potential exists to design an EGCG-based antimicrobial that simultaneously targets FabG and FabI. It has been suggested that simultaneously inhibiting two targets could reduce not only the frequency of resistant mutants, but also the extent of resistance conferred by single point mutations (Silver 2011). These properties illustrate the great potential of EGCG-based inhibitors that could be designed through structure based drug design, using the structure of NmFabI in complex with EGCG as a starting model.
Figure 5.13 - *N. meningitidis* FabI in complex with the inhibitor epigallocatechin gallate. (A-B) Epigallocatechin gallate (EGCG) (yellow) binds to *N. meningitidis* FabI in a similar position to the adenine ring of the co-factor NADH, forming hydrogen bonds with residues Cys63, Asp64, and Val65 (yellow dashed lines). (C) A 3D representation of the key interactions between EGCG and FabI. In addition to hydrogen bonds (blue lines), EGCG is stabilised by hydrophobic interactions with Val40, Val41, Ile92, Phe94, and Ile119 (silver clouds). (D) A 2D schematic representation of the interactions between FabI and EGCG. Hydrogen bonds are represented by green dashed lines; hydrophobic contacts are shown as semi-circular arcs.
CHAPTER 6 - GENERAL DISCUSSION AND FUTURE DIRECTIONS

The extent of antimicrobial drug resistance has become particularly prominent in *Neisseria gonorrhoeae*. A bacterium once routinely treated with a single course of penicillin, extensively drug resistant strains of *N. gonorrhoeae* have emerged, displaying high levels of resistance to all but a few remaining treatments. There are no vaccines available for prevention of *N. gonorrhoea* infections, thus current means of treatment remain antibiotic therapy, which will inevitably lead to increased resistance to these last remaining treatments. The emergence of extensively drug resistant *N. gonorrhoeae*, displaying resistance to multiple antimicrobials including front line extended-spectrum cephalosporin treatments, in many countries, including Australia, is testament to this (Unemo et al. 2012a, Unemo et al. 2012b, Lewis et al. 2013, Lewis 2014, Unemo et al. 2014). Antimicrobial resistance in *N. meningitidis* is not as prevalent as in *N. gonorrhoeae*, presumably as the development of vaccines targeting *N. meningitidis* have led to decreased antimicrobial treatment and thus less evolutionary pressure to develop such resistance. Nonetheless, the growing incidence of drug resistant *N. meningitidis* is also cause for concern, with at least one report of reduced susceptibility to the extended-spectrum cephalosporins ceftriaxone and cefotaxime in isolates from India (Nicolas 2007). The increasing drug resistance displayed by both these pathogenic bacteria demonstrates a clear need for the development of new antimicrobial treatments.

The FASII pathway of bacteria has garnered considerable interest as a target of novel antimicrobials, at least partly driven by an increasing trend of antimicrobial drug resistance. To address the issue of increasing antimicrobial resistance in *N. meningitidis* and *N. gonorrhoeae*, this study aimed to determine the three-dimensional structures of
Neisseria spp. FASII enzymes in order to provide a platform for rational structure-based drug design of new antimicrobial agents to target drug resistant N. meningitidis and N. gonorrhoeae. This study also aimed to characterise the interactions between the inhibitor EGCG and the FASII reductases FabG and FabI, for the development of novel compounds that simultaneously target both enzymes. In theory, such dual inhibitors would reduce the frequency of bacterial resistance against these compounds, as multiple and simultaneous mutations would be required to confer resistance (Silver 2011).

The structures of N. meningitidis AcpS, FabD, FabH, FabF, FabG, and FabI were determined by X-ray crystallography. AcpS, FabG, and FabI were also solved in complex with their respective co-factors, and additionally, FabI was solved in complex with triclosan, and EGCG, the first experimentally determined FabI:EGCG structure. Comparison of the crystallographic structures of N. meningitidis FASII enzymes to their homologues in other bacteria reveals that the active site architecture of these enzymes is highly conserved. This suggests that the catalytic mechanism is also conserved, and inhibitors that target these enzymes are likely to exhibit similar inhibitory activity against N. meningitidis FASII enzymes as those reported for other bacteria. Based on a review of the literature and structural comparison of N. meningitidis FASII enzymes to bacterial and human homologues, AcpS, FabF, FabG, and FabI appear to be the most promising targets of novel antimicrobial agents.

The role of AcpS at the beginning of the FASII pathway positions it as a highly desirable drug target, with inhibitors targeting AcpS, likely to block cell growth due to the toxic accumulation of apo-ACP, and inhibit fatty acid synthesis by preventing the formation the holo-ACP, the active form of ACP required to shuttle fatty acid substrates
between enzymes of the FASII pathway. However, the essentiality of AcpS in *Neisseria spp.* has not been determined experimentally. While published literature indicates *Neisseria spp.* possess no alternate phosphopantetheinyl transferase (PPTase) (Donadio et al. 2007), this would need to be confirmed to ensure the success of inhibitors targeting AcpS. If *N. meningitidis* or *N. gonorrhoeae* do possess other PPTase type enzymes, an inhibitor designed to simultaneously target AcpS and alternate PPTases, such as *B. subtilis* Sfp, like the AcpS inhibitor ML267, could be investigated. Such PPTase inhibitors may also be beneficial in combating or preventing any potential antimicrobial resistance that may arise due to homologous horizontal gene transfer with the PPTase genes from bacteria of another genus. Like AcpS, FabD needs to be further investigated in order to determine whether this enzyme is a valid drug target.

Knockdown models in *E. coli* suggest that FabD is essential for bacterial survival, yet current reported inhibitors, including the phenylbenzoic acid based compound reported by (Shen et al. 2010) and the aporphine alkaloid corytuberine, display poor antimicrobial properties (Lu et al. 2007, Shen et al. 2010, Sun et al. 2012). Whether this poor antimicrobial activity is due to inadequacies of the inhibitors, such as removal due to antimicrobial efflux pumps, or issues related to FabD as a drug target is yet to be determined, with the lack of quantitative data regarding the inhibitory activities of these molecules and other FabD inhibitors making it impossible to draw conclusions as to which may be the case. Until these issues are further investigated, FabD remains a less attractive drug target compared to other Fab enzymes, such as FabF or FabI.

Targeted alone, FabH does not appear to be a promising drug target, with deletion studies in wild type *E. coli* suggesting either AcpS, FabD or FabF are able to circumvent the necessity of FabH (Yao et al. 2012). Despite this, a number of antibacterial compounds that inhibit FabH have been reported, however whether these
have antibacterial activity solely due to inhibition of FabH or due to additional targets has not been established (Wang et al. 2013). Regardless of the uncertainty regarding the essentiality of FabH, targeting FabH and FabF simultaneously appears to be a promising option for development of novel antimicrobials. While the FabH and FabF inhibitors platencin and platensimycin have been shown to be potent FabF inhibitors, poor antimicrobial activity in *E. coli* as a result of efflux by the AcrAB–TolC efflux pump has led to the suggestion that their effectiveness is limited to Gram-positive bacteria. *N. meningitidis* and *N. gonorrhoeae* do not possess the AcrAB–TolC efflux pump found in *E. coli*. Instead, these bacteria are known to rely on the FarA-FarB, MtrC-MtrD-MtrE, MacA-MacB and NorM efflux pumps to export antimicrobial compounds (Rouquette-Loughlin et al. 2003, Kamal et al. 2007, Kamal et al. 2010), however whether these efflux mechanisms are able to export platencin and platensimycin remains untested. Furthermore, docking of both platencin and platensimycin to the active site of *Nm*FabF indicates no steric clashes or residue changes, which would prevent these inhibitors from binding, thus platencin, and platensimycin could possess significant antimicrobial activity against both *N. meningitidis* and *N. gonorrhoeae*, hence their MIC and IC$_{50}$ values warrant investigation. Moreover, *in vivo* studies in mice as well as Hela toxicity studies indicate platencin and platensimycin possess good pharmacological properties, however the ability of platensimycin to inhibit the human FASI complex, and inhibit rodent liver fatty acid synthesis *in vivo* (Wu et al. 2011) suggests platencin and platensimycin will need to be further modified in order to increase their specificity for bacterial FASII enzymes.

FabI is a well-established antimicrobial target, with the FabI inhibitors AFN-1252, CG400549, and MUT056399 currently undergoing clinical trials for the treatment of MRSA *S. aureus* infections, in addition to the wide spread use of the FabI inhibitor
triclosan in many antibacterial hygiene products. The MIC values of AFN-1252, CG400549 or triclosan against *N. meningitidis* and *N. gonorrhoeae* do not appear to have been investigated (Park et al. 2007, Karlowsky et al. 2009, Kaplan et al. 2012, Banevicius et al. 2013, Kaplan et al. 2013), however an MIC<sub>90</sub> of 0.25 µg/mL against *N. meningitidis* and *N. gonorrhoeae* has been reported for the inhibitor MUT056399 (Escaich et al. 2011), hence confirming the validity of FabI as a drug target for these bacteria. Superposition of MUT056399 and AFN-1252 bound SaFabI structures onto NmFabI, as well as NmFabI in complex with triclosan give no indication that these inhibitors would be less effective against *N. meningitidis* and *N. gonorrhoeae* than *S. aureus* assuming the molecules are not eliminated due to efflux. This is not an issue in regards to MUT056399, as indicated by an MIC<sub>90</sub> of 0.25 µg/mL against these species (Escaich et al. 2011). Despite the presence of a similar hydrophobic pocket to which triclosan, AFN-1252, MUT056399, and CG400549 bind FabI, these compounds appear to be ineffective for inhibition of FabG due to steric clashes. Yet as this hydrophobic pocket adjacent to the co-factor nicotinamide ring is present in both FabG and FabI, drug design in consideration of any potential clashes could result in potent inhibitors of FabG based on these compounds. However, such inhibitors may face resistance due to single point mutations as like those observed in FabI (McMurry et al. 1998, Stewart et al. 1999, Park et al. 2007, Escaich et al. 2011, Yao et al. 2013).

In contrast to the potent inhibitors targeting FabF and FabI, most reported inhibitors of FabG are either not accompanied by MIC values, or display poor antimicrobial properties. For instance, the inhibitor FG01 appears to be a potent inhibitor, with a reported IC<sub>50</sub> value of 0.02 μM against *Pseudomonas aeruginosa* FabG, yet no MIC or other assessments of antimicrobial activity has been reported (Cukier et al. 2013). Likewise, antimicrobial activity for chlorogenic acid and the related cinnamic acid
derived inhibitors have also not been reported. Of the FabG inhibitors with reported antimicrobial activity, EGCG, other green tea catechins, and the structurally similar chalcones butein, isoliquiritigenin, and 2, 2’, 4’-trihydroxychalcone are arguably the most potent. However, antimicrobial activity for these inhibitors is only observed in efflux negative *E. coli*, with no significant antibacterial activity observed against wild type *E. coli* and many other Gram-negative bacteria (Zhang et al. 2004, Tasdemir et al. 2006, Matsumoto et al. 2012). *N. meningitidis* and *N. gonorrhoeae* appear to be exceptions to this, with EGCG and the acylated EGCG derivative C16:EGCG exhibiting MIC values of 32 and 16 µg/ml respectively against both *N. meningitidis* and *N. gonorrhoeae* (Matsumoto et al. 2012), indicating that the efflux systems of these bacteria possess low affinity for EGCG, thus *N. meningitidis* and *N. gonorrhoeae* are incapable of rapidly exporting this compound. The enhanced antibacterial activity of the C16:EGCG compound synthesised by (Matsumoto et al. 2012) also demonstrates that EGCG can be effectively utilised as a lead compound for the development of more effective antibacterials.

As demonstrated in this thesis, EGCG binds to *NmFabI* in a position that overlaps with the binding site of the adenine portion of the FabI co-factor, NADH. This is consistent with a competitive mechanism of inhibition, as reported by Zhang et al. (2004). Preliminary enzymatic assays also confirmed EGCG inhibited *NmFabG* and *NmFabI:EGCG* with IC$_{50}$ values of 7 µM and 17 µM respectively. The fact that EGCG inhibits both FabG and FabI, two structurally and functionally similar reductases, suggests that EGCG binds these two enzymes at a similar location. Superposition of the *NmFabI:EGCG* and *NmFabG* structures (Figure 5.8) does not indicate any obvious steric clashes, and suggests that EGCG could form hydrogen bonds with *NmFabG*
residues Asn63 and Asp109, as well as hydrophobic interactions similar to those present in the *NmFabI:EGCG* complex.

The mechanism by which EGCG inhibits FabG is still unclear due to conflicting proposed mechanisms of inhibition, including suggestions that EGCG inhibits *E. coli* FabG in a competitive manner in regards to NADPH, a non-competitive manner in regards to co-factor, or possibly by covalent modification of FabG resulting in enzyme aggregation (Zhang et al. 2004, Li et al. 2006a, Li et al. 2006b). Based on superposition of *NmFabI:EGCG* and *NmFabG* structures, EGCG would appear to inhibit *NmFabG* in a competitive manner, however in the absence of mutagenesis, structural data, or further kinetic analysis, this is speculative, and such analyses would need to be conducted in order to conclusively determine the EGCG binding site and mechanism of inhibition.

A structure based drug design approach using EGCG as the lead compound could be utilised to design new inhibitors that display greater affinity for FabG and FabI, as well as increased specificity for these enzymes. Assuming EGCG binds FabG in a similar manner to FabI, one such approach may be to extend the EGCG based derivative further into the NADPH/NADH binding pocket by the addition of a cyclical motif resembling the ribose ring common to both co-factors. However, until the mechanism of inhibition of FabG by EGCG and the specific residues required for this inhibition have been elucidated, such structure based design approaches cannot be used.
Concluding remarks

Many aspects of the FASII pathway have been demonstrated to be valid drug targets, whilst other enzymes of the pathway, such as AcpS, FabD, and FabH, need further investigation to determine the usefulness of these enzymes as drug targets. The crystallographic structures of these enzymes reveal that their active sites, co-factor binding sites, and potential inhibitor binding sites are relatively unchanged in comparison to bacterial homologues. Based on the similarity of these enzymes to those reported in other bacteria, inhibitors targeting other bacterial FASII enzymes should also inhibit those found in *N. meningitidis* and *N. gonorrhoeae*. Further development of these inhibitors may lead to new classes of antibiotics to combat extensively drug resistant strains of *N. meningitidis* and *N. gonorrhoeae*. Additionally, the crystallographic structures of the *Neisseria* FASII enzymes may aid in the design of antibiotics specifically targeted at these increasingly drug resistant pathogens, with the potential for EGCG based inhibitors that simultaneously target *Neisseria spp.* FabG and FabI to not only treat drug resistant strains, but also reduce the development of antimicrobial resistance in *N. meningitidis* and *N. gonorrhoeae*. 


Wang, Y. and S. Ma (2013). Recent advances in inhibitors of bacterial fatty acid synthesis type II (FASII) system enzymes as potential antibacterial agents. ChemMedChem.


Chapter 2


Chapter 3


Chapter 4


Chapter 5


structural studies of NADH-dependent FabG used to increase the bacterial production of fatty acids under anaerobic conditions. Appl Environ Microbiol. 80(2):497-505


complex reveals a novel binding site distinct from the thyroxine binding site. Biochemistry 49(29): 6104-6114.


Chapter 6


Wang, Y. and S. Ma (2013). Recent advances in inhibitors of bacterial fatty acid synthesis type II (FASII) system enzymes as potential antibacterial agents. ChemMedChem.


Appendix 1 - Amino acid sequence alignment of FASII proteins. Sequence alignments of FASII proteins from *Neisseria meningitidis* (Nm), *Neisseria gonorrhoeae* (Ng), *Bacillus subtilis* (Bs), *Escherichia coli* (Ec), *Helicobacter pylori* (Hp), *Staphylococcus aureus* (Sa), *Streptomyces coelicolor* (Sc), *Streptococcus pneumoniae* (Sp), and *Vibrio cholerae* (Vc). Strictly conserved amino acid residues are highlighted blue with white text; similar amino acid residues are highlighted cyan. NCBI compatible accession numbers are provided alongside each aligned sequence. Sequence alignments were performed using the T-Coffee web server http://tcoffee.crg.cat/apps/tcoffee/index.html (Di Tommaso et al., 2011; Moretti et al., al., 2007). Figures were generated using the ESPript web server http://espript.ibcp.fr/ESPript/ESPript/ (Robert et al. 2014).
Note: *Bacillus subtilis* (Bs) possesses two FabH homologs, FabH1 and FabH2.
Note: *Bacillus subtilis* (Bs) possesses two FabH homologs, FabH1 and FabH2.
Note: *Streptococcus pneumoniae* (Sp) and *Vibrio cholerae* (Vc) genomes do not encode FabL type Enoyl-ACP reductases.
Appendix 2 - Molecular weights of protein standards used during SDS-PAGE. A representative image of the Bio-Rad Precision Plus Protein unstained standards used during SDS-PAGE to indicate the molecular weight of expressed and/or purified proteins. The molecular weight (in kDa) of each standard is shown next to the band.
Appendix 3 – Conference proceedings related to this work.

“Targeting the FASII reductases of Neisseria gonorrhoeae as novel treatment options to combat drug resistant gonorrhoea.” Presented at the 40th Lorne Conference on Protein Structure and Function (2015).

TARGETING THE FASII REDUCTASES OF NEISSERIA GONORRHOEA AS NOVEL TREATMENT OPTIONS TO COMBAT DRUG RESISTANT GONORRHOEA

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INTRODUCTION
Gonorrhoea is a sexually transmissible infection of major health concern due to high rates of disease and rapidly diminishing treatment options as a result of drug resistance. Reports of multi- and extensively resistant strains are of particular alarm. 114

The World Health Organisation has issued a Global Action Plan to minimise antimicrobial resistance in N. gonorrhoeae, calling for the development of new treatments as an urgent priority.

The USA CDC identified N. gonorrhoeae drug resistance in the top 3 of antibiotic resistance threats. With no ideal alternative treatment or vaccine available, it is feared that gonorrhoea will become untreatable, thus there is an urgent need for development of new antimicrobials.

The fatty acid synthesis type II (FASI) enzymes of N. gonorrhoeae, responsible for lipid biogenesis, represent an attractive target for the discovery of new antimicrobials. 114

The FASII reductases, FabG and FabI, are inhibited by epigallocatechin gallate (EGCG). 114

We have determined the structures of FabG and FabI, and that of FabI in complex with EGCG, NADH, and NADH-insoluble, providing detailed knowledge of the active and co-factor binding sites within both enzymes.

EGCG and NADH binding sites in FabI are highly similar in FabG, providing a basis for the design of inhibitors that simultaneously target both enzymes, and a strong platform towards the development of urgently needed antimicrobials.

METHODS

RESULTS

Inhibition of fatty acid elongation by EGCG
EGCG inhibits both FabG and FabI, preventing elongation of fatty acids. Preliminary enzyme assays confirmed EGCG inhibited FabG and FabI, with IC50 values of 0.1 and 2.1 μM, respectively. MIC values against drug resistant N. gonorrhoeae and N. meningitidis range from 0.12 to 32 μg/mL.

Possible interactions between FabG and EGCG
Superposition of the structures of FabG (cyan) and FabI (silver) in complex with EGCG (yellow) reveals little deviation in the secondary structure of the two enzymes (A), and possible hydrogen bonds (yellow dashed lines) between EGCG and FabG (B). The position of EGCG overlaps the binding site of the adenine portion of the NADH and NAD2+ in FabI (C) and FabG (D) respectively.

CONCLUSION
EGCG was found to bind to Neisseria FabI in a competitive manner with regards to the co-factor NADH. Superposition of the structures of Neisseria FabG, and FabI in complex with EGCG, indicates EGCG inhibits FabG in a similar manner; providing a basis for design of inhibitors that simultaneously target both enzymes. Mutagenesis and further enzymatic analysis is being conducted to clarify the mechanism of inhibition.

The FASI pathway of Neisseria spp.
Unlike the FASI pathway in humans, each reaction of the FASI pathway is catalysed by a distinct enzyme. In contrast to the FASI pathway found in E. coli, Neisseria spp. contain only a single enzyme to catalyse these reactions, making the FASI pathway of Neisseria an especially attractive target for development of antimicrobials.