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Structural and Functional Studies of Protein Targets at the Host-Pathogen Interface.

Samantha Capewell

PhD Thesis

University of Edinburgh.

2013
Declaration

I hereby declare that except where specific reference is made to other sources, the work contained in this thesis, to the best of my knowledge and belief, is the original work of my own research since registering for my PhD program in September 2009 and any collaboration has been indicated clearly. The thesis has been composed by the candidate and not been submitted, either in whole or part, for any other degree, diploma, or any other qualifications.

Samantha Capewell

May 2014.
Abstract

Ferric ABC Transporters.

Pathogenic bacteria have evolved specialised iron acquisition systems that allow them to effectively colonise a host. One of these systems is the ferric binding protein (Fbp) complex that is a member of the ATP-Binding Cassette (ABC) superfamily of small molecule transporters. The Fbp complex is made up of three-components (FbpABC) that transports ferric iron from the periplasm to the cytoplasm of many Gram negative bacteria. FbpA binds iron in the periplasm and transports it to the FbpB transporter complex that permeates the cytoplasmic membrane. Here the iron is actively transported by FbpB through the membrane that is powered by ATP hydrolysis catalysed by FbpC, the cytoplasmic ATPase.

*Burkholderia cenocepacia* is an opportunist pathogen that colonises the lungs of cystic fibrosis patients and is particularly resistant to antibiotic treatment. In this study the iron uptake system of *B. cenocepacia* strain J2315 is investigated. A putative FbpA from *B. cenocepacia* J2315 was expressed in the periplasm of *Escherichia coli* cells and the recombinant FbpA *B. cenocepacia* protein purified. The structural and electrochemical properties of native FbpA *B. cenocepacia* were investigated using UV Visible spectroscopy, spectro-electrochemistry, mass spectrometry and crystallographic techniques. It appears that FbpA *B. cenocepacia* is a novel member of the FbpA superfamily that selectively utilises citrate as an exogenous anion in ferric iron co-ordination. This is the first instance that a recombinant ferric binding protein has been documented as preferentially utilising citrate in this manner.

The putative ATPase from *B. cenocepacia* (FbpC *B. cenocepacia*) was also expressed in *E. coli* but it was found to be insoluble. A number of expression systems were tested but none were found to be successful in generating sufficient quantities of FbpC *B. cenocepacia* for structural studies.
Human β-defensin 2.

Despite daily contact with a range of microorganisms, mammals do not regularly succumb to pathogenic invasion. One reason is the presence of an important defence mechanism uses a reservoir of antimicrobial peptides (AMPs) that are expressed in eukaryotes as a means of innate immunity. The AMP superfamily is composed of over 900 members, displays broad structural and sequence diversity and is active against a wide range of bacteria, fungi and viruses. β-defensins are small (3-5 kDa), cationic peptides that display antimicrobial activity against a range of microbes and have also been shown to act as chemo-attractants (chemokines) within the adaptive immune system. In this study we obtained milligram amounts of pure human β-defensin 2 (HBD2) for functional studies by the development of a method for the rapid expression and purification of the recombinant peptide. A clone encoding a thioredoxin-HBD2 fusion protein was designed for the expression of soluble peptide in E. coli cells that was purified by simple affinity chromatography. The HBD2 peptide was cleaved from the fusion by an efficient protease step and further purified to yield pure HBD2. This recombinant HBD2 defensin was shown to be active against a Mycobacterium tuberculosis mutant strain.
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Introduction to ABC Transporters and Iron in Biology.

1.1 Metal Ions in Biology

It has been estimated that over 50% of all proteins will be metalloproteins (a protein that contains a metal co-factor) (1). Metal ions are crucial to biological systems, functioning as electron transporters, acting as co-factors and in the case of proteins, acting to increase the limited amino acid side chain reactivity of enzymes. Metal containing proteins are therefore of obvious research interest and any further understanding of their role can be considered a contribution (2).

1.1.1 Iron in Biology

Iron is an essential co-factor for many metabolic processes such as cellular redox regulation, forming bioorganic complexes and participating in critical respiratory processes. The concentration of iron required for growth by a bacterial cell is $10^{-7}$ M and as in humans the average cellular concentration of free $\text{Fe}^{3+}$ is ca. $10^{-24}$ M (3,4) the ability of a bacterial pathogen to acquire iron in a depleted environment is vital for successful colonisation of the host. As such bacterial acquisition of iron is considered to be a virulence factor. Bacteria have evolved specialised iron uptake machinery that are targeted toward extracting iron from the host environment and inability to acquire iron this way would result in the death of the bacterial cell. This designates inhibition of iron acquisition a desirable target for biochemical characterisation with an aim toward novel antibiotics.

This thesis will focus on the iron acquisition systems of two Gram-negative pathogenic bacterial strains: Neisseria gonorrhoea and Burkholderia cenocepacia.

1.1.2 Neisseria gonorrhoeae.

The bacterium N. gonorrhoea is an obligate human pathogen that causes the sexually transmitted disease, Gonorrhoea. Infection occurs through the lower genital tracts, mouth, and throat and on occasion via the eyeball. Infection with N. gonorrhoeae is rarely fatal,
however 10% of male cases and 80% of female cases are asymptomatic which can aid the unwitting transmission of the bacterium and also, if left untreated, cause complications such as pelvic inflammatory disease and sterility.

Antibiotic treatment for the disease was first available 10 years after the discovery of penicillin (1928) but within 30 years, penicillin and tetracycline resistant strains of *N. gonorrhoeae* had been isolated from human patients and are now common. By the 1970’s treatment had progressed and fluoroquinone antibiotics had become the first choice of treatment for Gonorrhoea, but by the 21st century strains resistant to this treatment had become prevalent. Since 2007 the treatment for Gonorrhoea infection usually consists of a single dose of a cephalosporin, a β-lactam antibiotic that is classified as a “last line of defence” as no other antibiotic treatments are currently available. However, two strains of *N. gonorrhoeae* (H041 & F89) have now been isolated and confirmed as having developed resistance to the cephalosporin antibiotics ceftriaxone and cefixime (5-6). As of yet there has not been a strain isolated with a genotype completely resistant to all available antibiotics, however the fact that all these genes have evolved (and rapidly) is cause for great concern. If a strain that has developed resistance to all the previously mentioned antibiotics is isolated then there will no longer be any available antibiotic treatment for that strain of the disease (7).

It is clear to see that the development of novel antibiotic compounds is crucial with regards to the *N. gonorrhoeae* bacterium. As iron acquisition is considered a virulence factor, understanding the mechanisms of iron acquisition in *N. gonorrhoeae* is of clinical relevance toward designing new antibiotic compounds.

### 1.1.3 *Burkholderia cepacia* complex (Bcc).

Historically referred to as members of the *Pseudomonads* but recently re-classified as *Burkholderia*, the Bcc refers to a sub-group of 17 genetically distinct but phenotypically similar bacteria that have all been isolated from the lungs/spittle of immuno-compromised human hosts (8-9). The Bcc species are part of the ubiquitous *Burkholderia* proteobacteria genus that currently lists more than 70 species found throughout the environment.
Bcc are not obligate human pathogens, instead the Bcc complex typically colonise plants and are considered beneficial in an agricultural environment as well as useful biotechnological strains thanks to large and dynamic genomes (10). However, they are capable of causing opportunistic nosocomial infections in humans and as such are currently classified at class II biosafety level. Famously, some strains of the Bcc can infect the lungs of cystic fibrosis (CF) sufferers, in some cases causing “Burkholderia syndrome” and death (11). Of the 12 Bcc strains, *B. cenocepacia* and *B. multivorans* are the most prevalent strains of the Bcc amongst CF patients although all genomovars of the Bcc complex have been isolated from CF patients. Infection with Bcc directly correlates with a poorer prognosis for CF sufferers and other immuno-compromised patients. Effective prophylaxis for affected people is difficult as Bcc bacteria are resistant to a variety of antibiotics, including trimethoprim (12) chloramphenicol (13), cationic peptides, aminoglycosides (kanamycin) and quinolones (9). The strains are of considerable clinical importance and as iron acquisition is considered a virulence factor, investigating the iron transport pathways of *Burkholderia cenocepacia* is of clinical relevance.

1.2 Iron Acquisition in Gram Negative Bacteria.

1.2.1 Regulation of Iron Uptake.

Iron is a highly reactive transition metal that in the aerobic environment is oxidised to Fe$^{3+}$. At this oxidation state it is highly insoluble in an aqueous environment at physiological pH (the concentration of free Fe$^{3+}$ is estimated to be $10^{-17}$ M far lower than the concentration required for cellular respiration) and as such cellular iron homeostasis is necessarily subject to tight regulation and cellular iron concentration levels are finely controlled. Monitoring cellular uptake of iron through the outer and periplasmic membranes is one such control mechanism and in Gram-negative bacteria this is globally regulated by ferric uptake regulator (Fur) proteins that respond to the cellular concentration of iron. Under iron-replete conditions FUR binds to ferrous iron and represses transcription of genes that encode for outer membrane iron transport proteins (14). When the cell is iron starved,
ferrous iron dissociates from FUR, which in turn dissociates from DNA and ceases to repress transcription of iron transport proteins (15). Since iron is usually in limited supply for pathogenic bacteria they respond by synthesising several iron up-take systems. These systems will be discussed below and all of the following systems that are discussed are regulated by FUR in the manner outlined above.

1.2.2 Iron Uptake Mechanisms of Gram-Negative Bacteria.

There are several mechanisms by which Gram-negative bacteria can import iron from an exogenous host/environment. The diversity in these systems reflect the differing iron sources that bacteria are able to utilise, such as directly binding iron binding proteins, haem sources or by using small, high affinity iron chelating molecules known as siderophores. Gram-negative bacteria possess an outer membrane (OM) that is considered impermeable to molecules greater than ~700 Da in size and as Fe³⁺ chelate complexes are generally larger transport requires a suitable membrane receptor. These receptors are usually highly specific, often with a Kₐ in the nM range for iron/iron-containing substrate, and actively translocate Fe³⁺ into the periplasm (16).

1.2.3 Iron Acquisition in N. gonorrhoeae.

*Neisseria* iron acquisition differs to that of other Gram-negative species as they lack siderophore secretion. As *Neisseria* do not secrete siderophores, the genus has evolved to rely entirely on iron sources from the human host. These include lactoferrin and transferrin haem and haemoglobin (17, 18, 19). The expression of a transferrin receptor complex has been proven necessary for *N. gonorrhoeae* infection of a male host which correlates with the presence of transferrin in semen, a known transmission pathway (20). The *Neisseria* genus are also capable of utilising xenosiderophores (siderophores secreted by other bacteria), as the bacterium expresses several outer membrane (OM) receptors capable of transporting these molecules allowing a system by which the bacterium is able to capitalise on the presence of other symbiotic bacteria in the host (21).
1.2.4 Iron Acquisition Mechanisms of *Burkholderia cenocepacia*

Infesting the human lung presents a challenge to pathogenic bacteria thanks to the presence of macrophages (white blood cells that are capable of recognising and destroying invading pathogens) in the alveolar passages and the scarcity of iron available. Iron homeostasis is regulated in a different manner in the lungs, compared to other parts of the body that are exposed to the outer environment, as the high partial pressure of oxygen increases the possibility of oxygen induced free radical damage. To this end, several iron sequestration systems have evolved to ensure that free iron is escorted from the environment and is either securely stored or immediately used in a functional role.

*B. cenocepacia* has evolved iron acquisition mechanisms that allow it to thrive in this environment. Unlike the genus *Neisseria*, *Burkholderia* synthesise and secrete four different siderophores, ornibactin, pyochelin, ceptabactin and cepaciachelin as well as utilising xenosiderophores (22). As of yet the specific molecular mechanisms that allow this pathogen to proliferate in the CF lung, but not healthy lungs have not been determined, but acquisition systems are up-regulated in pathogenic strains (23).

The iron sources that have been identified as a source for *B. cenocepacia* include ferritin, haemin, lactoferrin, and transferrin (24). It is known that the expression of ferritin, a 450 kDa globular protein capable of binding up to 4500 Fe$^{3+}$ ions, is up-regulated in CF lungs compared to healthy lungs. A ~ 70 fold increase in ferritin was measured in the alveolar lavage fluid of CF patients with no discernible increase in transferrin levels (25). Recently it has been determined that *B. cenocepacia* is able to utilise ferritin as a sole iron source and that this was mediated by proteolytic digestion of the ferritin complex suggesting that an ability to access an iron store as rich as ferritin maybe be a mechanism by which *B. cenocepacia* can colonise CF lungs (24).
1.3 Bacterial Iron Acquisition from Human Host Sources.

1.3.1 Transferrin Iron Sources

Human transferrin is an 80 kDa bi-lobal blood plasma glycoprotein that is made up of 4 subdomains, N1, N2, C1 & C2. Each subdomain (N1+N2, C1+C2) co-ordinates a single Fe$^{3+}$, resulting in a total of two atoms bound per fully holotransferrin protein. Each lobe requires a synergistic CO$_3^{2-}$ anion to complete co-ordination of ferric iron with a $K_d = 10^{23}$ M$^{-1}$ at pH 7.4 (26).

*N. gonorrhoeae* require expression of two outer membrane proteins for successful acquisition of iron from transferrin, identified as transferrin binding protein A (TbpA) and transferrin binding protein B (Tbp B) (27). TbpA is an 100 kDa OM protein with 22 transmembrane domains and an N-terminal periplasmic plug domain that is considered crucial for iron transport (28). The transmembrane domains conform to a membrane spanning C-terminal β-barrel with the N-terminal plug domain essentially folding up inside the β-barrel, effectively blocking the channel through the membrane. TbpA is crucial for the utilisation of transferrin iron sources, and it performs this in synergy with TbpB. Whilst TbpB itself is not essential for TbpA to function as a transporter, the protein acts to increase the efficiency of the transport process by discriminating between apo and holotransferrin forms (29-30). The exact mechanisms of iron release from transferrin have not been elucidated and this is due, in part, to the associated difficulties of working with membrane bound proteins such as TbpA and TbpB. The exact role of TbpB in acquiring iron from transferrin is not clearly defined as of yet but it is believed that TbpB is a surface exposed lipid modified protein with two distinct transferrin epitopes (31).

Recently, the structure of TbpA from *Neisseria meningitides* bound to human apo-transferrin was solved by small angle neutron scattering (SAXS) analysis and X-ray crystallography. Binding to TbpA is mediated exclusively through the electron negative C lobe of TF and the electron positive extracellular domains of the TbpA transmembrane pore. An unusually long TbpA N-terminal plug domain interacts directly with the Tf C$_1$ subdomain in combination with an extra cellular α-helix that inserts into the cleft between

18
the C₁ and C₂ domains. This forces Tf into a partially open conformation and the release of iron from the C-lobe binding site is a result of destabilisation. TbpB binds Tf via the C-lobe but distinct to the TbpA binding site and enforces selectivity for holo-Tf. (32-33)

1.3.2 Haem as an Iron Source.

As haem is the most abundant iron containing molecule in vertebrates (70% of total human iron content) it is a rich potential source of iron for bacterial pathogens and is widely utilised by them. Haem sources include haemopexin, haemoglobin, and albumin (34). The acquisition of haem iron by a bacterial cell can occur through a number of systems. In some cases haem, or a haem containing complex is recognised by a specific OM receptor and translocated into the periplasm before further transport into the cytoplasm. Other systems involve the synthesis and secretion of haemophores, which are small extracellular proteins expressed by Gram negative bacteria, that bind are capable of binding haem and shuttling it to a cognate OM receptor for translocation.

An example of a bacterial haem transport system is Haem acquisition system A (HasA). HasA is a haemophore that that is able to bind free haem/compete haem from haemoglobin and shuttle it to the bacterial OM receptor HasR (35). *Serratia marcescens* HasA is a 19 kDa protein that is required for uptake of haemoglobin-haem. An OM receptor, HasR is essential for haem uptake and acts in synergy with HasA to greatly increase the acquisition of haem from haemoglobin (35).

Upon binding to HasR, which has much lower affinity for haem than HasA, haem is then internalised via the receptor channel. In this case the haemophore is not internalised alongside its haem ligand and instead remains extracellular. Once haem has been transported into the periplasm it is bound by a periplasmic binding protein (PBP) that then delivers it to an ABC transporter complex located in the cytoplasmic membrane whereby it is further translocated into the cytoplasm. Once in the cytoplasm haem is either incorporated into proteins or is degraded as an iron source.
1.3.3 Siderophore Mediated Iron Acquisition.

Siderophores are small chelating molecules, secreted by bacteria that bind Fe$^{3+}$ with extremely high affinity. Enterobactin, a siderophore secreted by *E. coli* binds Fe$^{3+}$ with an affinity of $K_a = 10^{52}$ M$^{-1}$ and is able to compete iron from the iron binding proteins Tf and Lf (4). Over 500 siderophores have been isolated to date, and they are currently divided into five sub-groups; catecholates, hydroximates, carboxylates, heterocyclic compounds and mixed. These compounds are too large for diffusion through the cell membrane and so must be delivered to outer membrane (OM) receptors for active transport into the bacterial cytoplasm.

Uptake of siderophores is mediated through a two-gated system with a high affinity OM receptor as the first gate and an ABC transporter as the second. Several OM receptors have been identified and their structures solved revealing an overall similarity to the β-barrel transmembrane pore conformation of TbpA. Each monomeric OM receptor has 22 transmembrane domains with an N-terminal plug that occludes the translocation cavity and displays high affinity and selectivity for its appropriate siderophore ligand (36). Once the siderophore has been transported into the periplasm (via the “first gate”) it encounters the “second gate” and is acquired by a periplasmic binding protein, shuttled to the cytoplasmic membrane and transported to the cytoplasm by an ABC transporter. At this point iron dissociates from the siderophore/periplasmic binding protein complex and is transported via the membrane permease. The siderophore is thought to be recycled, and is not transported into the cytoplasm.

1.3.4 Ton B Dependent Iron Acquisition.

All of the previously discussed iron transport systems require the active transport of substrate across the outer membrane and into the periplasmic space. This comes at a metabolic cost to the cell and as there is no energy provision for active transport across the outer membrane, the energy requirement of trans-locating iron across the OM via the
receptors discussed above must be met by other means. In Gram negative bacteria this is performed by the FUR regulated expression of an inner-membrane complex of proteins named TonB, ExbB and ExbD that harness the proton-motive force of the cytoplasmic membrane (CM) to power the translocation of substrate through OM receptors. This type of transport is described as TonB dependant. (37-38)

Figure 1: Schematic representation of TonB dependant iron acquisition in Gram negative bacteria. Extracellular iron/ iron protein complexes bind to cognate OM receptors and are then transported into the periplasm. Provision of energy is provided by direct contact with the TonB complex that is tethered to the cytoplasmic membrane and spans the periplasmic space. Highly specific periplasmic binding proteins bind to the substrate and shuttle it to cognate ABC transporters for translocation into the cytosol.

TonB is a three domain protein that is anchored to the CM, ExbB and ExbD by its N-terminal domain and has a C-terminal domain that crosses the periplasm and makes direct
contact with OM receptors. All TonB dependent OM receptors have a conserved N-terminal motif referred to as the TonB box with the consensus sequence TITVTA that the C-terminal domain of TonB recognises. The mechanism by which the TonB complex operates is not yet understood, but the structure of the C-terminal domain of TonB in complex with OM receptors has been determined (39, 40).

![Figure 2: Structure of the OM receptors FhuA (A) and BtuB (B) in complex with C-terminal fragment of the CM protein TonB. (PDB code 1XX3 & 2GSK)](image)

The β-barrel of the OM receptors permease is coloured silver with the internal plug domains highlighted as green. The interacting C-terminal domain of the TonB complex is red in both cases.

The N-terminal plug domain of TonB dependent receptors occludes the β-barrel lumen when in the ground state, binds to the extracellular substrate and interacts with TonB. It is assumed that the interaction with TonB will enforce a conformational change that results in movement of the plug domain and an opening the transporter lumen (38).

### 1.4 ATP Binding Cassette (ABC) Transporters

Once substrate has translocated into the periplasm it must then be actively transported into the cytoplasm. TonB dependant iron transport in *N. gonorrhoea* requires the presence
of a second membrane transporter system known as an ABC transporter to shuttle iron through the periplasmic membrane.

ABC transporters are the largest superfamily of trans-membrane proteins currently identified and have been identified throughout eukaryote and prokaryote organisms. This family of carrier proteins shows great diversity in substrates and can be split into two distinct subclasses. The first contains complexes that import and export substrates across the extra and intracellular membranes of eukaryotic and prokaryotic organisms, whilst the second subgroup are involved in the maintenance of DNA \(41\). The ABC import/export transporters possess a canonical structure consisting of two transmembrane domains (TMD’s) and dimeric nucleotide binding domains (NBD’s) that facilitate active transport via the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). The stoichiometry of the complex is typically 2 x TMD: 2 x NBD and the hydrolysis of 2 x ATP molecules is required for a single translocation cycle. The TMD domains are functionally redundant in the absence of the partner NBD domains as ATP hydrolysis is essential for conformational changes that allow the translocation cavities to open and close. Whilst the NBD and TMD domains are distinct proteins an N-terminal loop belonging to the NBD domain is in direct contact with the cytoplasmic side of the TMD pore. As TMD proteins are membrane bound the NBD domains can be thought of as membrane associated. These transporters are able to translocate a diverse range of substrates such as amino acids, vitamins, divalent metal ions, peptides and lipids, into the cytoplasm and often against concentration gradients \(42, 43, 44\). Both prokaryotic and eukaryotic cells possess export ABC transporters but import complexes are the preserve of prokaryotic organisms and necessitate a third component called the periplasmic binding protein (PBP) (Figure 3).
Figure 3: Schematic representation of a prokaryotic ABC import transporter. The periplasmic binding protein (PBP) is green coloured and illustrated as in complex with the transmembrane dimer (TMD). Active transport of the substrate is powered by the cytosolic nucleotide binding domains (NBD) via ATP hydrolysis.

This monomeric protein acts as a high affinity scavenger within the periplasm, binding substrate and shuttling it to the periplasmic side of the associated transport TMD dimer (45).

ABC transporters share highly conserved amino acid sequences located in the NDB that pertain to the binding and hydrolysis of ATP and illustrate a common engine that drives this type of transport. There is no such primary sequence homology between TMD’s or PBP’s although conserved tertiary structure can be identified throughout both classes of protein.

1.4.1 Bacterial ABC Importers

There are currently several characterised bacterial ABC transporter systems and the x-ray crystal structures of the maltose, methionine and vitaminB12 transporters of E. coli and the molybdate/tungstate transporter of A. fulgidus and H. influenzae have been solved. Currently, these import transporters are divided into two distinct sub-classes that are dependent upon the TMD structure. These are defined as Type I importer folds and Type II importer folds. The maltose transporter (MalFGK2) and vitamin B12 transporter (BtuCD-F) are examples of type I and type II TMD folds respectively.
1.4.2 Type I ABC Importers.

The first observed structure of a type I transporter (otherwise known as small ABC importers) was reported when the x-ray crystal structure of the *Archaeoglobus fulgidus* molybdate/tungstate transporter (ModABC) was solved complete with periplasmic binding protein and tungstate transport substrate. Later, the MalF & MalG subunits of the *E. coli* maltose transporter (MalGFK₂) and methionine transporter (MetI) were captured (46, 47). Type I folds have, as observed so far, a minimum of 5 transmembrane (TM) helices per monomer which adopt varying concomitant conformations relative to ATP hydrolysis by the nucleotide binding domain. ModB, MalF and Mal G and MetI monomers each contain 6,8,6 and 5 TM helices respectively totalling 12,14 and 10 helices per trans-membrane channel. In general the substrates of these transporters are “smaller” molecules such as ions, sugars and amino acids as opposed to larger substrates such as peptides and metal chelate complexes (48). Unlike other type I systems MalGFK₂ displays dimeric asymmetry as the MalF TMD monomer possesses a cytoplasmic extension that has not yet been seen in any other transporter (P2, Figure 4).

![Figure 4: Examples of the Type I E. coli maltose ABC transporter (MalGFK₂) and the E. coli vitamin B₁₂ Type II ABC transporter (BtuCD-F).](image)

The difference in the number of transmembrane helices is visible in the x-ray crystal structures, with MalGF (PDB 3PUY) containing 5 from each TMD monomer, compared to 10 helices per TMD monomer in the BtuCD-F structure (PDB 2QI9).
Structural data of the transporter in the absence of the cognate PBP showed that the TMD and NBD domains adopt a closed conformation where the transmembrane lumen is closed to the periplasm and the NBD domains are open. In this conformation, the active site of the NBD dimer is open, and ATP is not bound. Binding of maltose binding protein stimulates a conformational switch in the TMD dimer, concomitant with ATP binding and closure of the NBD active sites. Conformational change in the TMD dimer results in the previously sealed “periplasmic gate” is opening and allowing the translocation of maltose from the PBP into the permease cavity. Hydrolysis of ATP, and the following dissociation of ADP + Pi is synonymous with opening of the NBD dimer and return to the resting state, i.e., closure of the TMD periplasmic gate (49-50).

1.4.3 Type II ABC Transporters

Type II importer folds (aka as large ABC transporters) are exemplified by the extensively characterised E. coli vitamin B₁₂ transporter (Figure 4) and contain 10 TM helices per TMD monomer, totalling 20 helices. Substrates of the type II transporters are usually larger in size than those of the type I transporters and include vitamins and metal chelates. The homologous Hemophilus influenzae importer, Hi1470/Hi1471 also displays the same 10 TM helices per monomer as the E. coli vitamin B₁₂ transporter (51). Substrate specificity toward vitamin B₁₂ is conferred entirely by the substrate specific PBP as the TMD structures caught bear no resemblance to the B₁₂ binding site. This has developed the idea that type II transporters provide an inert or “Teflon” passage of translocation through the periplasmic membrane whereby substrate specificity is provided entirely by the discerning periplasmic binding protein (48).

1.4.4 Transmembrane Domains and Nucleotide Binding Domains

Transmembrane domains are redundant in the absence of their counter part NBD domains so the study of these as a separate entity is pointless. Crystallographic studies have shown there is little primary sequence homology between TMB domains but there is conservation of structure and mechanism. All bacterial import TMD domains so far elucidated fall into the Type I or Type II transporter categories and all utilise membrane spanning α helices to
form a gated translocation pathway. Significant differences in amino acid sequence can be attributed to the differences in chemical characteristics of the various substrates that these complexes transport, and sequence homology is higher amongst homologous transporters of the same substrate.

The NBD domains of ABC transporters all possess a common architecture with highly conserved sequences that are ubiquitous throughout the entire ABC family irrespective of transporter substrate. Each transporter is in the possession of two NBDs that act as a dimer capable of binding up to two molecules of ATP.

Numerous structures of isolated NBDs available that reveal that the ATP binding sites are located at the dimer interface and residues from each monomer are required for hydrolysis to occur. Several conserved sequences are required for ATP hydrolysis. These are the Walker A GxxxxGK(T/S) consensus sequence (also known as the P loop), the Walker B (LxDEP) that contributes a catalytic base (E) that is essential for hydrolysis and the LSGGQ motif (commonly referred to as the ABC signature motif). Also, the D-loop (xLDx), H-loop (S/T-H-D/E) and Q-loop motifs are ubiquitous throughout NBDs and are involved in the co-ordination and stabilisation of nucleotide (43-52).

Another feature of some NBDs is the C-terminal regulatory domain, a ~100 residue extension that appears to function as a site for allosteric control of ATPase activity. This method of regulation, designated as trans-inhibition, has been documented in the type I M. acetivorans molybdate/tungstate transporter (ModBC) and the E. coli methionine transporter (MetNI) (42-53).

1.4.5 The Periplasmic Binding Proteins

Periplasmic binding protein (PBPs) describes a super family of soluble, cargo carrying proteins that are free in the periplasm of Gram-negative bacteria. PBPs are not specific to ABC transporters and as are members of transmembrane transport, chemoreception and signal transduction pathways. Classification as a PBP is based on the fact that these proteins have low primary sequence homology, but share highly conserved tertiary structure. Typically, a flexible hinge region that surrounds the ligand binding site,
sandwiched at the interface of the two domains, joins two globular domains. The structure of this hinge region has been used to classify the periplasmic binding proteins based on the range of structures currently available. Class I and II both have hinge domains that are connected by β-strands while those designated as class III possess a hinge region that is an α helix (54). Class II proteins include *N. gonorrhoeae* Ferric Binding Protein A (FbpA) that shuttles ferric iron from the outer to inner membranes. The two domains of FbpA are joined via a hinge region made up of two antiparallel β-strands and allows for a large degree of inter-domain movement around it. When apo, the protein is considered to be in an “open” form with the two globular domains positioned further apart and the ligand binding site more solvent exposed. Upon ligand binding the protein undergoes significant conformational change in which the two domains close around the molecule, facilitated by the mobile hinge region (Figure 5). This binding motion is what has led to this group of proteins being dubbed “Pac man” or “Venus fly trap” proteins and contributes to the high binding affinities that are characteristic (55).

### 1.5 Ferric Iron ABC Transport.

Ferric binding proteins (Fbp) are a sub-group of PBP’s that complex ferric iron from OM transporters and deliver it to the periplasmic membrane for transport into the cytoplasm. Characterised as monomeric, bilobal, globular proteins that are generally ~35 kDa in size and bind a single ferric iron with pseudo-octahedral geometry, these proteins coordinate iron with high specificity and affinity, usually with the aid of an exogenous, synergistic anion that completes the ion binding site alongside amino acid side chains.
1.5.1 Ferric Binding Protein from *N. gonorrhoeae*.

Recombinant FbpA *N. gonorrhoeae* was first purified from *E. coli* periplasmic extract in 1992 and has been extensively characterised in the twenty years since (56). FbpA *N. gonorrhoeae* is a periplasmic binding protein expressed by *N. gonorrhoeae* under iron limiting conditions (57). It is translated as an un-processed 37 kDa pre-peptide that includes a 23 amino acid N-terminal leader sequence targeting the pre-peptide for translocation into the periplasm (58). Upon translocation (presumably) via the SecA-dependant pathway, the N-terminal sequence is cleaved by a peptidase located on the periplasmic side of the cytosolic (inner) membrane allowing post-translational folding into “mature” FbpA protein.

The structure of FbpA *N. gonorrhoeae* has been solved (PDB 1D9Y) and reveals a monomeric 34 kDa, 308 amino acid protein that binds Fe$^{3+}$ in synergy with an exogenous PO$_4^{3-}$ anion resulting in a *holo* complex of FbpA-Fe-PO$_4$ with a 1:1:1 stoichiometry. FbpA *N. gonorrhoeae* binds Fe$^{3+}$ with pseudo octahedral geometry via a glutamic acid carboxylate, an imidazole nitrogen from a histidine side chain and two tyrosinate oxygen atoms (Table
The six ligands required for complete coordination are fulfilled by a mono-dentate $\text{PO}_4^{3-}$-and a water and result in a $K_a$ for Fe$^{3+}$ of $10^{18} \text{ M}^{-1}$ (Figure 6) (59).

Figure 6: Fe$^{3+}$ sequestration by FbpA N. gonorrhoeae showing the iron co-ordination motif that is made up of the amino acids His9, Glu 57, Tyr 195,196 and a mono-dentate synergistic phosphate anion. A water that is not visible completes octahedral co-ordination. Image rendered from the X-ray crystal structure PDB 1D9Y.

Historically, FbpA N. gonorrhoeae has been referred to as "bacterial transferrin" throughout the literature and was once considered a member of the transferrin superfamily. This is not an unwarranted comparison as both FbpA and transferrin sequester Fe$^{3+}$ and require an exogenous synergistic anion to complete the first co-ordination shell. In both proteins the amino acid side chains that contribute to the direct sequestering of Fe consist of a two tyrosines, a histidine and a glutamate (FbpA N. gonorrhoeae) or an aspartate residue (transferrin). In contrast to FbpA N. gonorrhoeae, transferrin completes the pseudo-octahedral co-ordination sphere of Fe$^{3+}$ by utilising a bi-dentate carbonate anion rather than a phosphate anion and water molecule as described in FbpA N. gonorrhoeae. Both proteins have a common function in their respective organisms in that they both bind ferric iron with high affinity and shuttle it to cognate membrane receptors, maintaining tight control over iron toxicity and preventing possible participation in harmful Fenton chemistry (55).

Whilst FbpA N. gonorrhoeae has become the archetypal FbpA, the first solved structure of a ferric binding protein was that of the homologue FbpA Heamophilus influenzae. The x-ray crystal structure of recombinant FbpA H. influenzae was solved in 1997 (PDB 1MRP) (60) and reveals mature FbpA H. influenzae to be a 34.5 kDa protein that co-ordinates ferric iron
with a single exogenous phosphate anion, a water and four hard side chain ligands in an identical fashion to FbpA *N. gonorrhoeae*. Maximum peak absorbance exhibited by the LMCT band of holo FbpA *H. influenzae* is visible at 483nm and is highly similar to that of FbpA *N. gonorrhoeae* (481 nm). FbpA *N. gonorrhoeae* and *H. influenzae* share 86% amino acid sequence homology. Although, we will not elaborate here, FbpA *N. gonorrhoeae* and *H. influenzae* are able to bind and transport metal clusters and metals other than iron (61-62).

Since 1997 the structures of 6 other FbpA proteins from various organisms (including *N. gonorrhoeae*) have been solved (this data is summarised in Table 1). All known ferric binding proteins co-ordinate ferric iron in pseudo-octahedral fashion, contributing up to six ligands in the form of amino acid side chains. Most of these proteins also employ an exogenous, synergistic anion that is essential for high affinity binding and can vary depending upon organism of origin.

Table 1 lists the structures available and the manner in which the holo protein co-ordinates ferric iron. Structural characteristics of these ferric binding proteins, alongside phylogenetic sequence analysis of putative ferric binding proteins have been used to determine classification systems for this sub-group of periplasmic binding proteins (63-64).
Table 1: Currently available ferric binding protein structures from Gram negative bacteria (Dec 2012)

<table>
<thead>
<tr>
<th>PDB</th>
<th>Organism</th>
<th>Exogenous Ligand</th>
<th>Res (Å)</th>
<th>LMCT λ max</th>
<th>Fe Binding</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D9Y</td>
<td><em>N. gonorrhoeae</em></td>
<td>PO₄³⁻</td>
<td>2.20</td>
<td>481 nm</td>
<td>Y195, Y196, H9, E57, PO₄³⁻ &amp; H₂O.</td>
<td>N/A</td>
</tr>
<tr>
<td>3TYH</td>
<td><em>N. gonorrhoeae</em></td>
<td>Oxo-copper</td>
<td>2.10</td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>1R1N</td>
<td><em>N. gonorrhoeae</em></td>
<td>Tri-nuclear oxo</td>
<td>1.74</td>
<td></td>
<td>(65)</td>
<td></td>
</tr>
<tr>
<td>1O7T</td>
<td><em>N. gonorrhoeae</em></td>
<td>Hafnium</td>
<td>1.65</td>
<td></td>
<td>(62)</td>
<td></td>
</tr>
<tr>
<td>1XC1</td>
<td><em>N. gonorrhoeae</em></td>
<td>Zirconium</td>
<td>1.51</td>
<td></td>
<td>(66)</td>
<td></td>
</tr>
<tr>
<td>1MRP</td>
<td><em>H. influenzae</em></td>
<td>PO₄³⁻</td>
<td>1.60</td>
<td>483 nm</td>
<td>Y195, Y196, H9, E57, PO₄³⁻ and H₂O.</td>
<td>(60)</td>
</tr>
<tr>
<td>3OD7</td>
<td><em>H. influenzae</em></td>
<td>PO₄³⁻</td>
<td>2.10</td>
<td></td>
<td>(67)</td>
<td></td>
</tr>
<tr>
<td>3OBD</td>
<td><em>H. influenzae</em></td>
<td>Fe³⁺</td>
<td>3.09</td>
<td></td>
<td>(67)</td>
<td></td>
</tr>
<tr>
<td>1D9V</td>
<td><em>H. influenzae</em></td>
<td>N/A [apo]</td>
<td>1.79</td>
<td></td>
<td>N/A</td>
<td>(68)</td>
</tr>
<tr>
<td>3KN8, 3KN7</td>
<td><em>H. influenzae</em></td>
<td>PO₄³⁻ (mutants)</td>
<td></td>
<td></td>
<td>(67)</td>
<td></td>
</tr>
<tr>
<td>1NNF</td>
<td><em>H. influenzae</em></td>
<td></td>
<td>1.90</td>
<td></td>
<td>(69)</td>
<td></td>
</tr>
<tr>
<td>1QW0</td>
<td><em>H. influenzae</em></td>
<td>PO₄³⁻</td>
<td>1.10</td>
<td></td>
<td>(70)</td>
<td></td>
</tr>
<tr>
<td>1QVS</td>
<td><em>H. influenzae</em></td>
<td>PO₄³⁻</td>
<td>2.10</td>
<td></td>
<td>(70)</td>
<td></td>
</tr>
<tr>
<td>1XVX</td>
<td><em>Yersinia</em> enterocolitica</td>
<td>N/A</td>
<td>1.53</td>
<td>513 nm</td>
<td>E62, Y198, Y199, H14 &amp; D144</td>
<td>(64)</td>
</tr>
<tr>
<td>1Y4T</td>
<td><em>Campylobacter jejuni</em></td>
<td>N/A</td>
<td>1.80</td>
<td>440 nm</td>
<td>Y146, R105, H14, Y203, Y202, Y 15</td>
<td>(63)</td>
</tr>
<tr>
<td>1Q35</td>
<td><em>Mannheimia haemolytica.</em></td>
<td>Apo</td>
<td>1.20</td>
<td>N/A</td>
<td>Formate:R136, D178, R179, Y142, Y198, Y199, R101 &amp; E11.</td>
<td>(71)</td>
</tr>
<tr>
<td>1S1</td>
<td><em>M. haemolytica</em></td>
<td>N/A</td>
<td>1.45</td>
<td>419 nm</td>
<td>Y142, Y198 &amp; Y199.</td>
<td>(72)</td>
</tr>
<tr>
<td>1S0</td>
<td><em>M. haemolytica</em></td>
<td>CO₃</td>
<td>1.35</td>
<td>419 nm</td>
<td>Y142, Y198, Y199, Bidentate CO₃.</td>
<td>(72)</td>
</tr>
<tr>
<td>1Y9U</td>
<td><em>Bordetella pertussis</em></td>
<td>apo</td>
<td>1.39</td>
<td>N/A</td>
<td>N/A</td>
<td>(73)</td>
</tr>
<tr>
<td>2OWT</td>
<td><em>B. pertussis</em></td>
<td>CO₃²⁻</td>
<td>2.40</td>
<td>416 nm</td>
<td>Y143, Y198, Y 199 + bidentate CO₃.</td>
<td>N/A</td>
</tr>
<tr>
<td>2OWS</td>
<td><em>B. pertussis</em></td>
<td>Oxalate (x2)</td>
<td>1.42</td>
<td>428 nm</td>
<td>Y200, Y143, 2 x bidentate oxalate.</td>
<td>N/A</td>
</tr>
<tr>
<td>1XVY</td>
<td><em>Serratia marcescens</em></td>
<td>N/A</td>
<td>1.74</td>
<td>510 nm</td>
<td>Citrate binding; His 11, Glu 59, Ser 176 and Tyr 196.</td>
<td>(64)</td>
</tr>
<tr>
<td>2PT2</td>
<td><em>Synechocystis</em> 6803</td>
<td>N/A</td>
<td>2.00</td>
<td>411 nm</td>
<td>Tyr 55,185,241 &amp;242, His 54</td>
<td>(74)</td>
</tr>
</tbody>
</table>
All FbpA proteins currently characterised possess a peak maximal absorbance between 400-520 nm that is a result of a ligand to metal charge transfer (LMCT) when the in *holo* form. This is most likely due to interaction between the metal and tyrosinate oxygen atoms, and the diversity of the LMCT absorbance maxima in differing protein reflects the differing iron binding environments. The peak maximal absorbance for each of the proteins is illustrated in Table 1. In general, maximal absorbance of the carbonate binding proteins is at the lower end of the range at 416 and 419 nm for *holo B. pertussis* and *M. haemolytica* respectively. *Holo B. pertussis* with two oxalate anions possesses a LMCT peak maximum at 428 nm and the anion independent *C. jejuni* at 440 nm. *N. gonorrhoeae* and *H. influenzae* have a near identical maximum at 481 nm and 483 nm respectively, which corresponds with their homologous iron binding sites. *Holo Y. enterocolitica* displays a red shifted maximum absorbance of 513 nm when compared to the other anion independent FbpA *C. jejuni*. The LMCT band of ferrous iron binding *Synechocystis* is visible at 411 nm.

### 1.5.2 Iron release from FbpA at the Receptor Interface.

The mechanism by which iron is transferred from Fbp to the TMD permease is not understood as of yet. Ferric iron binding proteins, such as FbpA and transferrin co-ordinate ferric iron with strong binding affinities ($K_d = 10^{-18}$ M and $10^{-23}$M$^1$ respectively) that must be overcome to facilitate the release of iron from the binding protein to the cognate receptor.

The $K_d$ of FbpA *N. gonorrhoeae* for Fe$^{3+}$ is estimated to decrease by 12 orders of magnitude if Fe$^{3+}$ is reduced to Fe$^{2+}$ and so it can be supposed that reduction of iron may play a role (75). The affinity of transferrin for Fe$^{2+}$ is estimated to be at least 14 orders of magnitude lower than that of Fe$^{3+}$, which is consistent with a model reductive release of iron from transferrin (76).

The Fe$^{3+}$/ Fe$^{2+}$ reduction potential of FbpA *N. gonorrhoeae* has been determined by Optically Transparent Thin Layer Cell (OTTLE) potentiometric titrations and established at -300mV which is within the range of physiologically available nicotinamide adenine dinucleotide (NAD) reductases (59). The redox potential of transferrin for Fe$^{3+}$, measured by the same technique, is considerably more reducing at -526 mV but was measured to be
200 mV more oxidising when bound to its receptor at endosomal pH (5.4) (75). Acidification of transferrin when taken up into the endosome has also been shown to significantly lower the Fe³⁺/Fe²⁺ redox potential of the protein putting it in range of endogenous reductases, although this method of iron release is not applicable to bacterial TbpA/TbpB uptake as transferrin is not taken up by the cell in this case. In the case of a Fbp, it is unlikely that significant pH change would occur at a receptor interface in the periplasm (77).

As the bacterial outer membrane is permeable to molecules < 700 Da then the periplasm is a reflection of the chemical environment that the cell is in. Physiologically abundant anions vary in differing environments and as Fbps are capable of facile interchange of anions whilst remaining bound to iron the role that variation of synergistic anion plays was called into question.

The effect of differing synergistic anions upon the redox potential was investigated by creating FbpA N. gonorrhoeae Fe³⁺ + X complexes (whereby X is representative of different physiologically available anions). Arsenate, oxalate, pyrophosphate, citrate and nitrilotriacetate (NTA) loaded FbpA Fe Fe³⁺ were used in further OTTLE potentiometric experiments and resulted in a 50 – 150 mV oxidative shift in comparison to the wild type protein. The measured E₁/₂ values for each complex, in decreasing potential order, are as follows - NTA (-186 mV)~oxalate (-186 mV)> citrate (-191 mV)> pyrophosphate (-212 mV)> arsenate (-251 mV)> phosphate (-300 mV). The effective binding constant of each of the variant synergistic anion complexes was also determined and found to vary from 4.2x 10¹⁸ M⁻¹ to 3.7x10¹⁷ M⁻¹ (75). From these findings it can be said that exchanging the synergistic anion of FbpA can lower the reduction potential and therefore the metabolic cost of reduction is also lowered.

An alternate theory to the reductive release of iron is the possibility of a conformational change induced by docking with the TMD domains that mechanically disrupts the binding site and forces release of iron. When the maltose transporter MalFGK₂ was caught in complex with substrate loaded PBP, the substrate binding site had been disrupted by a cytoplasmic loop of the TMD dimer and the substrate itself had moved into the transmembrane lumen (47). This is consistent with a mechanical disruption model, or at
least with model that partially consists of conformational change of the PBP when in complex with cognate receptor. It should be noted that the maltose ABC transporter is type I as typically are iron transporters and so tend to possess fewer transmembrane domains than type II complexes. Furthermore, the dissociation constant of MBP for maltose (1.2 μM)\(^{(78)}\) is significantly lower than that of FbpA for iron potentially reducing the energy required to force conformational change in the substrate binding protein. Finally, transport of maltose is unlikely to be as highly regulated as iron when taking into consideration that free Fe\(^{3+}\) is likely to be immediately hydrolysed/sequestered else could participate in damaging chemical reactions. Lower maltose regulation is reflected in lower binding constant and should serve to highlight that mechanical disruption may not be a universal model for substrate translocation in ABC transporters and maybe substrate dependant.

To date the question of how, and in what redox state, iron is transferred from Fbp to the TMD permease and through into the cytosol has not been answered. Further to this, despite the identification of many sub-classes of FbpA from various organisms there has been a distinct lack of biochemical and biophysical characterisation that may help determine the actual mechanism of transport. With the exception of FbpA \textit{N. gonorrhoeae}, only structural data is available with regard to the 6 other characterised FbpA proteins that are currently available, no biochemical/biophysical data is available. Further biochemical and biophysical characterisation of other FbpA proteins is required to provide further insight into the mechanistic properties of these proteins.

**1.6 Ferric Binding Protein C: Nucleotide Binding Domain from \textit{N. gonorrhoea}.**

The x-ray crystal structure of the \textit{N. gonorrhoeae} FbpC NBD ATPase in complex with Ca\(^{2+}\): ATP has been solved and reveals a homo-dimer \((43)\). The first 240 amino acids consist of typical ATPase topology and contain sequence motifs that are conserved throughout NBD proteins. The Walker A (also known as the P-loop or phosphate binding loop) consensus sequence GXX\(X\)XGK(T/S,) ,whereby X denotes any of the standard 20 amino acid, is between Gly\(^{37}\) and Thr\(^{44}\). The FbpC Walker A sequence is GASGCKT. K\(^{43}\) is considered
crucial to ATP binding and interacts with the γ and β phosphates of ATP via three hydrogen bonds.

Figure 7: The X-ray crystal structure of FbpC reveals a homo dimer caught with an ATP molecule (yellow) in each active sites (PDB 3FVQ (43)). The individual protein monomers are coloured green and pink. The N-terminal ATP binding regions can been seen as distinct domains compared to the C-terminal domain that manifests as a β-barrel consisting of both FbpC dimers.

The Walker B (R/K XXXX G XXXX L HHHH D, whereby H denotes a hydrophobic residue.) and LSSGQ motifs are also identified. Interestingly, the H loop His 197 and Walker B Glu 164 residues of the active site are swung away from the γ phosphate of ATP in the solved structure of FbpC (Figure 8: Active site of FbpC in complex with ATP and Ca2+ in the pre-hydrolysis state. Interaction between the H-loop histidine residue and the γ phosphate of ATP has been observed in other NBD structures and is proposed to stabilise the transition state of ATP hydrolysis (79). In the case of FbpC is it suggested that Ca2+ acting as the divalent metal cofactor has in fact hindered ATP hydrolysis by causing the unproductive conformation of the catalytic H-loop. 0.2M Ca2+ was used in the crystallisation buffers and so the effect of Ca2+ compared to the cofactor Mg2+ was unexpected.
**Figure 8:** Active site of FbpC in complex with ATP and Ca2+ in the pre-hydrolysis state. The supposed catalytic residue His197 is swung away from the Y-phosphate of ATP:Ca2+ resulting in a loss of hydrolysis that effectively locks the NBD dimer in a closed conformation.

A further point of interest that was revealed upon solving the structure was the discovery of an 110 amino acid long C-terminal domain (residues 242-352) that contains two oligotide binding folds (OB fold) similar to that seen in the maltose transporting MalK structure. This domain has been observed in other NBD proteins, but is not universally conserved in the manner as the N-terminal ATP binding NBD segment is. Unusually, the two monomers undergo a domain swap that has not been observed previous in ATPase proteins of this type. A cavity is formed at the base of the C-terminal domains and three residues from each monomer result in an ordered six histidine cluster that has the potential to form a metal binding site (Figure 9). Examples of ABC transporters using substrate as an allosteric regulation mechanism have been documented in both the *E. coli* methionine (*80*) and molybdenate/tungsten transporters (*81*).
Figure 9: The C-terminal domain swap and histidine cluster (red) of FbpC *N. gonorrhoeae*. The C-terminal domain is viewed perpendicular and beneath the membrane orientation of the protein, and shows the potential for a histidine gateway that substrate must pass through prior to entering the cytoplasm. The motif is made up of histidine residues from both NBD monomers. Monomer 1 is shaded pink and monomer 2 is green.

The molybdate transporter possesses a similar C-terminal extension to that of FbpC and upon oxyanion binding at the interface of the monomers confers a trans-inhibition state. Essentially, upon binding WO$_4^-$ the molybdate NBD is locked into a conformation that renders the nucleotide binding sites inaccessible. Furthermore a homologue of the molybdate transporter without the C-terminal regulatory domain does not display trans-inhibition in the same assay conditions. Trans-inhibition of the methionine MetNI ABC transporter complex via allosteric C-terminal regulation has also been documented. It therefore follows that the histidine cluster within the C-terminal domain of FbpC may function as an allosteric control mechanism (53).
Chapter 2  Introduction to Antimicrobial Peptides and Human β Defensins.

2.1 Defensins: Peptide Antimicrobial Agents of the Innate Immune System

Antimicrobial peptides (AMPs) are expressed in a variety of species including humans and plants. Their function is to provide a level of static immunity against the constant onslaught of bacterial invasion that we face on a daily basis. Currently the AMP family is of considerable interest clinically as ever more antibiotic resistant strains of bacteria evolve.

Defensins are characterised by their small size, cationicity and distinct disulfide bond (S-S) connectivity arising from conserved cysteine amino acid sequence motifs. They are organised into three separate sub-classes: α, β, & θ that are dependent on both the number of conserved cysteine residues and distinct disulfide bridging patterns within the peptide. It has been found that α and β defensins both possess 6 cysteine residues but with differing S-S connectivity, whilst θ are cyclic peptides and have only 5 cysteine residues (102). α and β defensins have been identified and isolated from human sources and although θ defensin genes have been identified in the human genome, the only primate source was purified from macaque monkeys (102-103-104). Over 400 open reading frames (ORFs) have been identified in the human genome as possessing defensin signature cysteine motifs, although currently only 10 defensin proteins have been identified in vivo (105).

2.1.1 Discovery of the first Defensins.

The first human "defensins", originally named Human Neutrophil Peptides (HNP) were discovered in a bactericidal extract in 1966, but it was not until the 1980's that these peptides were extracted from azurophil granules of human neutrophils (106-107). It was from Ganz et al in 1985 that the term “defensin” was first used to describe three peptides, HNP 1, 2 and 3 that had been successfully isolated. These were found to be homologous to six peptides previously isolated from rabbit granulocytes, and to also possess almost identical sequence homology with the exception of their N-terminal amino acid. HNP 4 was also isolated from neutrophils, whilst HNP 5 & 6 were discovered to be expressed in
epithelial cell found in the crypts of Leiberkuhn (intestinal glands)\(^{108-109}\). All of these antimicrobial peptides have a conserved disulfide bond motif with identical connectivity of Cys1-6, Cys 2-4 and Cys 3-5.

In 1991 a 38 AA peptide possessing antimicrobial activity was isolated from bovine tracheal mucosa, named Tracheal Antimicrobial Peptide (TAP) followed by 13 other peptides from bovine neutrophils \(^{110}\). These peptides possessed differing disulfide bond connectivity to those previously isolated, and defined a new class of defensin; \(\beta\)-defensins. The disulfide bond connectivity of \(\beta\)-defensins is Cys1-5, Cys 2-4 and Cys 3-6.

\[
\begin{align*}
\alpha & \quad X_{(1-2)}C^1XC^2RX_{(2-3)}C^3XXXEXXXC^4XXXGXXXXC^5C^6X_{(1-4)} \\
\beta & \quad X_{(2-10)}C^1X_{(5-6)}G/AXC^2X_{(3-4)}C^3X_{(9-13)}C^4_{(4-7)}C^5C^6X_{(n)} \\
\theta & \quad GXCRCXCXRGXCRCXCXR \quad \text{(cyclic)}
\end{align*}
\]

**Figure 10: Consensus Sequence of Defensin Peptides.** \(X\) represents any amino acid. Disulfide bond connectivity is represented by lines and the ordering of the cysteine residues is representative of their chronology within the peptide sequence.

The first human \(\beta\)-defensin (HDB1) was isolated from the blood plasma of patients suffering with renal disease in 1995 \(^{111}\). This novel peptide was found to share the same \(\beta\)-defensin disulfide bond connectivity as TAP and bovine neutrophil \(\beta\)-defensins leading to classification as the first human \(\beta\)-defensin. Two years later HBD2 was purified from the skin lesions of psoriasis sufferers using an unusual bacterial affinity chromatography method which was followed in 2001 by HDB3 using the same methodology \(^{103,112}\).
Synthetic HBD4 was both expressed and synthesised later in the same year after the Human Genome Project allowed analysis and identification of numerous potential defensin genes on various chromosomes. This report uses Human β-defensin 2 as a model defensin peptide, and so despite the variety of antimicrobial peptides, the focus of this work will be on HBD2 and other β-defensins (113).

2.1.2 β-defensins and Expression.

Unlike human α-defensins that are expressed primarily in neutrophils and some Paneth cells, β-defensins are expressed in epithelial cells. HBD1 is predominately expressed in the epithelial cells of the urinary and respiratory tracts, HBD2 in skin, respiratory and gastrointestinal tracts and HBD3 can be found throughout the epithelial cells and some other cell types also. HBD4 expression has been detected in the testis and epididymis, along with inducible expression *in vivo* in keratinocytes.

The individual expression levels and regulation characteristics are highly variable from peptide to peptide. HBD1 expression is constitutive although expression can be increased by exposure to bacterial immunogenic agents. HBD2, 3 & 4 are expressed at a basal level throughout the epithelia until up-regulation is stimulated by either bacterial infection or pro-inflammatory stimuli. Expression of all three of these peptides is induced by pro-inflammatory agonists such as lipopolysaccharide (LPS), Tumour Necrosis Factor α (TNFα), Interleukin-1β (IL-1β), IL-1α and IFN-γ and for HBD2 this response is mediated via the transcription factor NF-κβ(114).

Modulation of expression is attributed to a class of membrane receptors called Toll-like Receptors (TLR's) and these are critical in the regulation of defensins throughout various tissues. TLR-2 and TLR-4 are thought to be involved in the control of HBD2 expression in response to stimulus by the agents mentioned previously (115-116). However, the complexities of pathways involved in this process are poorly understood and despite considerable research the mechanisms of defensin regulation are not still not defined.
2.2 β-defensins link Innate and Adaptive Immunity.

In addition to antimicrobial activity, a second function as chemokines was discovered when it was established that an α-defensin-containing extract was able to chemoattract monocytes (117). β-defensins 1,2 & 3 have also been proven as chemotactic for immature dendritic cells (iDC) and memory T-cells (CD4+) (118). The chemotactic activity of HBD1 & HBD2 was found to be mediated through a G-Protein Coupled Receptor (GPCR) named CC Chemokine Receptor 6 (CCR6). Competition assays with a known CCR6 chemokine ligand, MIP-3α (Macrophage Inflammatory Protein 3α), that also shares with HBD2, structural features at the tertiary level and similar charge distribution despite low sequence homology further suggest that β-defensins are active ligands for CCR6.

The role of β-defensins as chemokines is further expanded by the ability to attract macrophages and monocytes, cells which do not express CCR6. Another GPCR, CCR2 is expressed in neutrophils, macrophages and monocytes and has recently been identified as the mediator of this interaction (119). HBD2, HBD3 and their mouse orthologues were able to chemoattract CCR2 transfected HEK293 cells (Human endocrine), an effect which was knocked out by incubation with the chemokine ligand of CCR2 MCP-1 (Monocyte chemoattractant protein-1). That defensins act as chemokines implies that these peptides are not only static members of innate immunity, but also play a role in the recruitment of an adaptive response to pathogenic invasion. The two systems of mammalian immunity, innate and adaptive, have traditionally been thought of as separate but slightly overlapping entities. Innate immunity often is a rapid, static and clinically symptomless response to immunogenic stimulus that is non-specific and confers no long lasting protection to the host. Defensins, as antimicrobial peptides, are an example of innate immunity with their broad bactericidal activity and constitutive expression. Adaptive immunity refers to the “specific” pro-inflammatory adaptable response that produces long term protection against various pathogens. Unlike innate immunity, the adaptive response is a long-lasting event that continues for weeks after infection and creates our “immune memory” of antibodies against specific antigens. The ability of defensins to chemoattract leukocytes
points to a role in both innate and adaptive immunity, with the chemotaxis of iDC cells as a possible inducer of antigen specific immune response.

2.2.1 Structure-Function Relationships

Since β-defensins have low primary sequence homology but highly conserved secondary and tertiary structural features, structure-function relationships are considered to be key to understanding how these peptides function. Several human β-defensins have been identified but the disulfide bond connectivity has only been confirmed experimentally for HBD1-4. Sequence alignment analysis revealed that, with the exception of the 6 characteristic cysteines, the only conserved residues are Glu^{10} and Glu^{21} (numbered according to HBD1). Although there is low sequence similarity between β-defensins, the x-ray crystal and NMR solution structures revealed similarities in their tertiary structures \((120-121)\). To date the X-ray crystal structures of synthetic and native human β-defensins 1, 2 & 3 have been solved as well as various bovine, murine and avian defensins \((122)\). A common structural feature is the “defensin-like” fold, a central motif that consists of 3 anti-parallel β-strands stabilised by the 3 canonical disulfide bonds (Figure 11). In β-defensins this motif also includes an α-helix formed by the N-terminal segment of the peptide, and constrained by a disulfide bond.

\[\text{Figure 11: NMR solution Human β-defensin 2 (PDB code 1FD3). (A) The “defensin-like” fold, three core β-strands are shown as antiparallel arrows, stabilised by three disulfide bonds (yellow). The beta defensin N-terminal α-helix is also visible and constrained by the C1-C5 disulfide bond. (B) Surface representation of HBD2 highlighted to show the distribution of charge across the peptide (cationic residues are red). HBD2 has an overall charge of +6.}\]
All defensin peptides possess an overall cationic charge (ranging from +4 to +11) that is inferred by a high level of lysine and arginine residues. An overall cationic charge is a characteristic found throughout the AMP family and is thought to play a role in the mechanism by bacterial cells are killed.

Originally it was thought that the structure of defensins conferred by the disulfide bond connectivity was essential for antimicrobial activity. However, studies have shown that mutagenesis of the cysteine residues to alanine had no effect upon anti-microbial activity (123–125). In fact a linear HBD3 with all cysteine residues replaced with α-aminobutyric acid (Figure 12) displayed the same antimicrobial activity as the wild type (124).

![Figure 12](image)

**Figure 12:** (A) L-α-aminobutyric acid. (B) L-Cysteine

Recently, the potency of HBD1 was shown to increase significantly under reducing conditions (126). HBD1 is considered to be the most ubiquitous defensin of its class but also displays, when oxidised, the least antimicrobial activity until reduced. In light of these findings the role of the conserved cysteine residues may be to function in a redox regulation mechanism over antimicrobial activity.

Despite the long standing label of “antimicrobial peptides” the mechanism by which defensins kill has not been clearly defined although electrostatic and hydrophobic interactions are thought to play a role. Bacterial cell membranes are generally negatively charged due to the presence of anionic components such as lipotechoic acid and LPS (lipopolysaccharide), allowing cationic AMPs to differentiate between these and neutrally charged eukaryotic cells (127). The only extensive structure-function analysis of a β-defensin was reported when 26 single point mutations of HBD1 were analysed for antimicrobial activity. Significant biological differences clustered around residues Arg29,
Lys$_{31}$, Lys$_{33}$, and Lys$_{36}$ with HBD1 alanine mutants at these positions showing reduced lethality toward *E. coli* and indicating that the C-terminal of HBD1 is associated with antimicrobial activity (120). A seven amino acid C-terminal truncation of HBD1 displayed a loss of antimicrobial activity, while conversely the C-terminal *GKAKCCK* fragment was partially active. Mutation of the two cysteine residues to alanine or serine also abolished killing activity, and indicates that the C-terminal cysteine residues are either directly essential for lethality or play a regulatory role (126).

Once an electrostatic interaction has occurred between defensin and antimicrobial peptide there are three current models that hypothesise mechanisms of killing. Defensins are in fact amphipathic with distinct areas of cationic and hydrophobic residues. The “torodial pore” model suggests that after interacting with the bacterial membrane electrostatically, hydrophobic patches are able to perturb the lipid membrane, disrupting integrity and ultimately causing death. The “carpet” model focuses solely on cationic charge and theorises that the peptides utilise electrostatic interaction to carpet the bacterial membrane until it is “choked” to death (128). The third model involves quaternary organisation of defensins to form oligomers. Known as the “barrel-stave” model, the formation of oligomers would result in permeating transmembrane pores causing the cells to leak to death. The mechanism which applies to β-defensins has not been determined, although some claims of higher order oligomerisation have been reported (129).

### 2.2.2 Defensins are Chemokines.

Whilst it was established that antimicrobial activity is independent of S-S connectivity it was still thought that it might be required for chemotaxis. However, Taylor et al demonstrated that cysteine to alanine mutants of HBD3 and its murine orthologue Defb14 are not chemotactic for cells expressing CCR6 (124). Interestingly, chemotaxis activity of HBD3 and Defb14 was still intact after iodoacetamide alkylation of the cysteine sulphur and activity could be restored in peptides with a cysteine in the fifth position (130). Alanine is a smaller residue that cysteine and is also un-charged as it possesses methyl R-group. Conversely, iodoacetamide alkylation of cysteine causes a shift in R-group charge to positive so the difference in chemotaxis activity could be attributed to charge. In an
attempt to localise activity, a series of overlapping Defb14 fragments were assayed for both chemotaxis and bactericidal activity. None of the fragments were chemotactic, indicating that a single epitope is not responsible, but all the fragments, and in particular ones from the N-terminal were potent bactericides.

Taylor concludes that S-S bonding is not critical for antimicrobial or chemotactic activity in HBD3, but this is not a view universally shared. 26 single point mutants of HBD1 were also assayed for chemotaxis of CCR6-transfected HEK293 cells and several key residues were identified. Asp\(^1\) – Ser\(^8\), Lys\(^{22}\), Arg\(^{29}\) and Lys\(^{33}\) alanine mutants resulted in reduced chemotaxis and are notably located in the N terminal of the peptide or adjacent to it (131).

CCR6 has only one known “natural” ligand besides the defensins HBD1-3, the chemokine MIP-3\(\alpha\) (Macrophage Inflammatory Protein). As mentioned previously, competition assays using defensin or MIP3\(\alpha\) as inhibitors led to decreased chemotaxis in the presence of the other ligand. Comparisons between β-defensins and MIP3\(\alpha\) have therefore been made to understand the binding mechanism between these peptides and CCR6. Like β-defensins MIP3\(\alpha\) is expressed as a pro-peptide with an N-terminal sequence that is subsequently cleaved to leave an 8 kDa peptide that is expressed constitutively in a range of tissues. Expression has been found to be up regulated in keratinocytes by bacterial presence and MIP-3\(\alpha\) appears to possess greater antimicrobial activity than either HBD1 or 2 as well as discernable cationic patches (132-133). There is no significant sequence similarity, but some subtle structural similarities have been identified between HBD1 and MIP3\(\alpha\) and HBD2 and MIP-3\(\alpha\). Sequence analysis revealed the presence of a DCCL amino acid motif towards the N-terminus of the peptide. This motif is conserved throughout the CC chemokine family and is believed to be involved in binding and activation of chemokine receptors. HBD2 also has an N-terminal DPV/DPVTCL sequence which could be thought to mimic this motif(134). Furthermore, a 13Å groove present in the face of MIP3\(\alpha\), formed by an N-loop and β2-β3 hairpin turn is also found as a narrow 9Å wide feature on the face of HBD1 & HBD2. It is suggested that this motif, and the size difference may contribute to MIP3\(\alpha\) possessing greater affinity for CCR6 as a ligand.
2.3 G-Protein Coupled Receptors (GPCR’s)

GPCR’s are one of the largest, protein families in humans with more than 30% of current drug targets focusing on signal transduction events carried by members (135). There are currently over 800 estimated GPCR’s in the human genome divided into 6 groups A-F, with group A subdivided into 19 classes (136, 137). Like defensins, GPCR’s do not have a high level of sequence homology, but do have a distinct structural similarity. It is this feature that lends itself to another name by which these proteins are known, the 7 transmembrane (7TM) receptors. These receptors are integral transmembrane receptors helices that span the bi-layer membrane of eukaryotic cells. They are able to transduce a huge number of signals across cell membranes via two main signalling pathways, cAMP or phosphatidylinositol. Typically GPCR’s have an extracellular N-terminal domain, followed by 7 transmembrane helices and an intracellular C-terminal which functions as a site for activation of an associated G protein through GDP/GTP exchange. Although the mechanism of signal transduction is not well understood, when an extracellular ligand stimulates the GPCR it is thought that it causes a conformational change in the receptor, facilitating guanine nucleotide exchange and G-protein activation. For most GPCR’s the extracellular stimulus could be one of many ligands (138).
Figure 13: Schematic representation of typical GPCR structure. GPCR proteins are eukaryotic transmembrane receptors that span the bi-lipid cell membrane. Known as the 7TM (transmembrane domain) transporters, they typically consist of 7 α-helical domains connected by cytoplasmic and extracellular loops. The N-terminus is located on the extracellular side of the membrane, while the C-terminus is cytoplasmic and interacts with the G-protein complex that gives this receptor its name. G-proteins form a hetero-trimeric complex consisting of α β & γ subunits and trigger an intracellular signal cascade upon signal transduction by the receptor protein.

2.3.1 CCR6: A member of the CC Chemokine Receptor Family.

CCR6 is a GPCR exclusively expressed on the surface of memory T cells, B cells and iDC cells and the only known receptor for the chemokine CCL20 and conversely CCL20 is its only chemokine ligand (139). As mentioned previously β-defensins are also active ligands for CCR6 despite low sequence homology, leading to the hypothesis that structural characteristics are crucial to the functional overlap between peptides. The biology of CCR6 has been extensively researched with expression detected in various tissues and cell types. As CCR6 is expressed on the surface of leukocyte cells, and in particular B cells, T cells and iDC cells, has led to the suggestion that CCR6 and its ligands are a link between innate and adaptive immunity. Understanding the molecular details of the interaction between HBD2 and CCR6 would be of great benefit. CC chemokine receptors share conserved cysteine residues located in the extra cellular domains, a feature which CCR6 also shares.
Mutational studies resulted in reduced surface expression when cysteines located in the first and second extracellular loops where replaced with serine. Investigation of the theory that only the extracellular domains are involved in ligand binding was carried out when CCR6/CCR5 chimeras were assayed for chemotactic activity. Replacing the extracellular domains of CCR6 with those of CCR5, on an individual or whole level, resulted in a total loss of chemotactic ability by MIP-3α stimulation. This finding implied that ligand binding in CCR6 is dependant on whole protein conformation and not a single specific epitope.

In summary, considerable research has focused upon defensins and their roles in human immunity, but as of yet structural analysis has been hindered by an inability to produce milligram quantities of peptide for analysis. Therefore to carry out research on defensins and their interaction partners, the first step is to express and purify significant quantities of pure defensin. However, defensins are antimicrobial, and are therefore toxic toward bacterial expression hosts, rendering traditional recombinant techniques of little use. Despite this, in our lab, recombinant HBD2 has been expressed effectively in BL21 [DE3] E. coli as an insoluble N-terminal keto-steroid isomerase (KSI) fusion. This method results in isolation of the protein within inclusion bodies, preventing possible antimicrobial activity during expression. However, this method of protein production is labour intensive, time consuming, and results in relatively low peptide yield (1mg per L culture).

HBD2 was originally purified from the skin lesions of psorasis sufferers using a bacterial affinity column (103). Unfortunately details of the chromatography were never published and have not been replicated by another group. Furthermore, quantities of human psoriasis scales are not easy to obtain. Defensin-antibody fusions have also been employed to express and purify HBD3 and its murine orthologue MBD14 (140). Another soluble HBD2 expression system has recently been achieved using a thioredoxin fusion technique which has also been used to prepare mouse beta defensin 1 (mBD1), and human-β-defensins 26 and 27 (HBD26 and HBD27) from E. coli cell free extracts (141). The use of thioredoxin fusions to improve protein solubility has been documented and vectors encoding the tag are commercially available, although the cellular mechanism that allows increases solubility is not clearly defined (142). Reported yields of mature peptide, using thioredoxin fusions are 140mg/L for mBD1, 138mg/L and 129mg/L for HB26 and HBD27.
– although these yields should be taken with care since they relied on the Bradford assay. It is also not clear if these yields were garnered from shake flask or fermentation cultures. Given the technical difficulty and time consumption of KSI fusion expression, and relatively low yields of mature peptide, attempts were made to express and purify soluble defensin peptides as thioredoxin fusions with an efficient protease release site.

2.4 Aims

The aims of this project are:

1. To analyse the B. cenocepacia J2315 genome, identify Fe^{3+} ABC transporter operons and to clone the operons into vectors for recombinant expression in E. coli.

2. Characterise the putative B. cenocepacia ABC transporter subunits (FbpA and FbpC) using UV-Vis spectroscopy, elemental analysis, enzyme kinetics, X-ray crystallography, and mass spectrometry.

3. Investigate the electrochemical properties of the periplasmic binding protein, to determine if redox chemistry plays a role in the release of iron from the protein:metal complex.

4. To prepare recombinant FbpC from N. gonorrhoeae and B. cenocepacia and investigate the role of the ordered histidine motif that was revealed by the x-ray crystal structure of FbpC N. gonorrhoeae.

5. To develop a rapid and efficient method for the soluble expression of HBD2 using E. coli as an expression strain and standard shake flasks as expression methodology.

6. Successfully purify HBD2 from an E. coli expression vector within a “normal” purification time frame and in the least complicated manner possible. This incorporates development and purification optimisation of the new HBD2 construct, cleavage steps and finally purification of the HBD2 peptide.
Chapter 3  Materials and Methods.

3.1 General Methods and Buffers

All reagents were purchased from Sigma unless otherwise noted. Restriction enzymes were purchased from New England Biolabs.

3.1.1 Bacterial Cell Lines

BL21(DE3) Chemically competent cells  
One Shot® TOP10 Chemically Competent E. coli  
DH5α E. coli

3.1.2 Escherichia coli Culture Media.

All culture media was 0.22µM filtered, UV treated and autoclaved at 121°C prior to use.

Luria Bertani (LB) was used as growth media in all cases unless otherwise stated. LB was composed of: 10g/L Peptone, 5g/L yeast extract and 10g/L NaCl and pH was adjusted to 7.5 using NaOH.

SOC Media: 20g/L Peptone, 5 g/L Yeast Extract, 0.58g/L NaCl, 0.185g/L KCl, 2.3g/L MgCl₂, 1.36g/L MgSO₄, 3.6g/L Glucose. The pH was adjusted to 7.5 with NaOH. Stocks were stored at 4°C.

2YT: 16g/L Peptone, 10g/L Yeast Extract, 5g/L NaCl and the pH was adjusted to 7.5 with NaOH.

LB Agar: 8.75g bacto-agar/ 250ml H₂O was dissolved by autoclaving and stored at 4°C until further use.

Terrific Broth: 12 g/L Tryptone, 24 g/L Yeast Extract made up and autoclaved. Plus 4 mL/L Glycerol, 2.31 g/L NaH₂PO₄, Na₂HPO₄ made up separately and added to the above mix after autoclaving, and at the point of use.
**Overnight Express™ Instant TB Medium** (Merck Biosciences): Media was made up as per the manufacturers instructions.

3.1.3 Antibiotic stocks.

All antibiotic stock solutions were sterilised by 0.22 μM filtration and stored at 4°C for up to 7 days.

Ampicillin sodium salt (Fischer BioReagents) was dissolved in dH₂O and used to make up 100 mg / mL stock solutions.

Kanamycin sulphate stock solutions were made up in water at 30 mg / mL.

Chloramphenicol antibiotic stocks were made up at 50 mg/ mL in 70% ethanol. Stocks were stored at -20°C.

3.1.4 *Escherichia coli* Competent Cell Transformation.

2μL mini-prepped plasmid DNA was added to an aliquot of chemically competent cells (volume dependent on cell strain) and incubated on ice for 20 minutes. The cells were heat shocked at 42°C for 30 seconds and 80μL SOC media was added. The cells were incubated at 37°C for 1 hr. before selection of transformants by spreading on LB agar plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight.

3.1.5 Preparation of Plasmid DNA Stocks

All plasmid DNA was prepared using the QIAprep® Spin Miniprep kit (Qiagen) following the protocol provided. Plasmid stocks were stored frozen at -20°C

3.1.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Protein samples were prepared for SDS PAGE as follows:

SDS Loading Buffer, 2 x: 10ml
Tris-HCl (1.5 M, pH 8.8) 3 mL
Glycerol (100%) 4 mL
SDS (10%) 2 mL
2-β-mercaptoethanol 800 µl
Bromophenol blue. 4 mL

Unless otherwise stated, all SDS PAGE gels were homemade using the following protocol and the BioRad gel system. Gels were loaded with sample and the BioRad gel tank was filled with 1 X Tris, acetic acid, EDTA buffer (TAE) and electophoresised for 50 – 60 mins at 200 V, 180 mA.

**Separating gel (15% v/v acrylamide)**

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<tr>
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</tr>
<tr>
<td>Acrylamide (30%)</td>
<td>7.5 mL</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>150 µL</td>
</tr>
<tr>
<td>APS (50 mg/mL)</td>
<td>300 µL</td>
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<tr>
<td>TEMED</td>
<td>20 µL</td>
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**Stacking gel (4% v/v acrylamide)**

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</tr>
<tr>
<td>TEMED</td>
<td>15 µL</td>
</tr>
</tbody>
</table>

**Preparation of Whole Cell Extracts for SDS PAGE analysis:**

1 mL of cell culture was collected by centrifugation in a bench top microfuge. The supernatant was discarded and pelleted cells were resuspended in 400 µL SDS Loading Buffer and then boiled for 5 minutes. Cell debris was collected by a brief microfuge spin and 10-15 µL sample was loaded into the gel wells.

**Cell Free Lysates and Purified Protein Samples:**

20 µL of sample was mixed with 20 µL SDS loading buffer and boiled for 5 minutes. 10-15 µL of sample was loaded into gel wells.
3.1.7 4-12% NuPAGE® Novex® 4-12% Bis Tris Precast Gels.

Pre-cast gradient gels were purchased from Invitrogen and used according to the manufacturer’s instruction. Briefly, gels were electrophoresised in an Invitrogen Xcell Surelock® Gel Tank using 1 x 2-(N-morpholino)ethanesulfonic acid (MES) running buffer at 200 V for 35 minutes (or until dye front ran to the bottom of the gel).

3.1.8 Agarose Gel Electrophoresis

1% Agarose gel recipe (100ml volume):
- 1 x Tris-acetate-EDTA (TAE) buffer
- Agarose
- Ethidium Bromide (10mg/mL)

DNA was electrophoresised at 100V for (typically) 1 hour.

3.1.9 Sanger Di-Deoxy Sequencing of Expression constructs

All plasmid DNA was prepared for sequencing reactions by purification using the Qiagen QIAprep® Spin Miniprep kit. The Big Dye method was employed in all cases.

5µL plasmid DNA
2µL Terminator 3.1 buffer
2µL Big Dye® Terminator
1µL Forward/ Reverse pET (T7) primer.

Thermal Cycle (x 25)
- 94°C for 30 seconds
- 50°C for 30 seconds
- 60 for 240 seconds

Sequencing was then performed by the GenePool (UoE) commercial sequencing service.
3.2 Materials and Methods: Ferric ABC Transporters

3.2.1 Construction of pET28a FbpA B. cenocepacia J2315 Expression Vector.

A 755bp synthetic gene encoding the gene BCAL1092 was purchased from Genscript with 5’ Ncol and 3’ XhoI restriction sites encoded. The gene was codon optimised for expression in E. coli. The gene was delivered as a pUC57 vector and was used to transform DH5α cells and the plasmid DNA extracted via Qiagen Mini-Prep Kit.

The gene insert was restricted from plasmid backbone by restriction digestion. 43 µL plasmid DNA, 1 µL Ncol, 1 µL XhoI and 5 µL 10 x NEB buffer 3 were incubated at 37 °C for 2 hours before 1% agarose gel electrophoresis to separate the excised 750bp fragment from the pUC57 plasmid back bone. DNA corresponding to the correct size fragment was extracted from the agarose gel using the QIAQuick DNA Extraction Kit from Qiagen.

For the expression vector, pET28a plasmid DNA was restricted using Ncol and XhoI in the manner outlined above. The plasmid backbone was extracted and gel purified. Vector and insert fragments were ligated for 5 minutes on ice according to the Roche Rapid Ligation Kit, before transformation into One Shot® TOP10 Chemically Competent E. coli cells. Colonies were cultured in 5ml LB kanamycin media, before plasmid DNA extraction and sequencing and restriction digestion to confirm successful ligation of insert. This plasmid was named pET28a FbpA J2315.

3.2.2 Construction of pETHISTEV FbpC B. cenocepacia J2315 Expression Vector

A 774 bp synthetic gene encoding the gene BCAL1090 was purchased from Genscript with 5’ Ncol and 3’ XhoI restriction sites encoded. The gene was codon optimised for expression in E. coli. The gene was delivered as a pUC57 vector and was used to transform DH5α cells and the plasmid DNA extracted via Qiagen Mini-Prep Kit.

The gene insert was restricted from plasmid backbone by restriction digestion. 43 µL plasmid DNA, 1µL Ncol, 1µL XhoI and 5µL 10 x NEB buffer 3 were incubated at 37 °C for 2
hours before 1% agarose gel electrophoresis to separate the excised 774 bp fragment from the pUC57 plasmid back bone. DNA corresponding to the correct size fragment was extracted from the agarose gel using the QIAQuick DNA Extraction Kit from Qiagen.

For the expression vector, pETHISTEV plasmid DNA was restricted using Ncol and Xhol in the manner outlined above. The plasmid backbone was extracted and gel purified. Vector and insert fragments were ligated for 5 minutes on ice according to the Roche Rapid Ligation Kit, before transformation into One Shot® TOP10 Chemically Competent E. coli cells. Colonies were cultured in 5ml LB kanamycin media, before plasmid DNA extraction and sequencing and restriction digestion to confirm successful ligation of insert. This plasmid was named pETHISTEV BCAL1090.

3.2.3 Construction of pETHISTEV FbpC N. gonorrhoeae Expression Vector.

FbpC N. gonorrhoeae was amplified from parental pET28a plasmid DNA by PCR.

Oligonucleotides:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FbpC HISTEV Forward</td>
<td>5’ ACCATGGGGGGCCGCC 3’</td>
</tr>
<tr>
<td>FbpC HISTEV Reverse</td>
<td>5’ AAGCTTTTATCCGGGAAGAAC 3’</td>
</tr>
</tbody>
</table>

PCR Protocol:

Two TaqBead™ Hot Start Polymerase (Promega) beads were dissolved in 22 μL H₂O, 1 μL template DNA (pET28a FbpC N. gonorrhoeae), 1 μL FbpC HISTEV Forward primer (10 μM) and 1 μL FbpC HISTEV Reverse primer (10 μM).

Thermal Cycle:

95°C for 5 minutes
95°C for 30 seconds
55°C for 30 seconds (x 25)
72°C for 2 minutes
72°C for 10 minutes
The PCR reaction was analysed by 1% agarose gel electrophoresis and corresponding PCR product was gel purified using the QIAQuick DNA Extraction Kit from Qiagen. The PCR product was then polyadenylated by the addition of another TaqBead™ Hot Start Polymerase followed by incubation at 95°C for 5 minutes.

The adenylated PCR product was ligated into pGEM T-easy cloning vector (Promega). 50ng pGEM T-easy vector, 1 μL Rapid Ligase (Promega), 3 μL PCR product and 5 μL 2 x Rapid Ligase buffer were incubated at room temperature for 1 hour before transformation into E. coli JM109 competent cells and selection on LB-Xgal agar.

Several colonies were screened for successful ligation by restriction digestion. Successful ligations were confirmed by DNA sequencing. At this point the FbpC gene insert was excised from pGEM T-easy by sequential restriction digestion with NcoI and HindIII (respectively) and then ligated into the expression vector pETHISTEV that had been previously restricted with the same enzymes. 1 μL pETHISTEV backbone vector and 3 μL FbpC gene insert were mixed with 1 μL T4 DNA Ligase (NEB) 1μL 10 x T4 ligase buffer and 4 μL H2O and incubated at room temperature for 1 hour. Ligation samples were then transformed into One Shot® TOP10 Chemically Competent E. coli cells and selected for on LB agar media. Single colonies were picked into 5ml LB media and incubated overnight at 37°C. These were used to prepare plasmid DNA and successful insertion of the FbpC gene into the plasmid was confirmed by restriction digestion analysis. The new FbpC expression vector was named pETHISTEV FbpC.

3.2.4 Construction of FbpC N. gonorrhoeae Δ111 amino acid C-terminal Truncation Expression Vector.

A FbpC N. gonorrhoeae Δ111 amino acid truncation was designed to be amplified from parental pET28a plasmid DNA by PCR.

Oligonucleotides:

<table>
<thead>
<tr>
<th>FbpC Truncation Forward 5’</th>
<th>GAGATATACCCATGGGGGCCG</th>
<th>3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>FbpC Truncation Reverse 5’</td>
<td>CTCGAGTCATCGCCGATAAACAG</td>
<td>3’</td>
</tr>
</tbody>
</table>

PCR Protocol:
Platinum® Pfx DNA polymerase (Life Technologies) was used in a 50 μL reaction volume with 5 μL 10 x pfx buffer, 1 μL MgSO₄, 1.5 μL dNTP mix (NEB) 1 μL forward primer, 1 μL reverse primer, 1 μL template DNA (mini-prepped plasmid stock), 0.4 μL Pfx polymerase and 48.1 μL H₂O.

22μL H₂O, 1μL template DNA (pET28a FbpC N. gonorrhoeae), 1μL FbpC HISTEV Forward primer and 1 μL FbpC HISTEV Reverse primer.

**Thermal Cycle:**

94°C for 5 minutes

94°C for 15 seconds

58°C for 30 seconds (x 25)

68°C for 1 minutes

68°C for 10 minutes

The PCR reaction was analysed by 1% agarose gel electrophoresis and corresponding PCR product was gel purified using the QIAQuick DNA Extraction Kit from Qiagen. The PCR product was then polyadenylated by the addition of a single TaqBead™ Hot Start Polymerase followed by incubation at 95°C for 5 minutes.

The adenylated PCR product was ligated into pGEM T-easy cloning vector (Promega). 50ng pGEM T-easy vector, 1 μL Rapid Ligase (Promega), 3 μL PCR product and 5 μL 2 x Rapid Ligase buffer were incubated at room temperature for 1 hour before transformation into E. coli JM109 competent cells and selection on LB-Xgal agar.

Several colonies were screened for successful ligation by restriction digestion. Successful ligations were confirmed by DNA sequencing. At this point the gene insert was excised from pGEM T-easy by double restriction digestion with Ncol and Xhol and then ligated into the expression vector pET28a. 1 μL pET28a backbone vector and 3 μL gene insert were mixed with 1 μL T4 DNA Ligase (NEB) 1μL 10 x T4 ligase buffer and 4 μL H₂O and incubated at room temperature for 1 hour. Ligation samples were then transformed into One Shot® TOP10 Chemically Competent E. coli cells and selected for on LB agar kanamycin media. Single colonies were picked into 5ml LB media and incubated overnight.
at 37°C. These were used to prepare plasmid DNA and restriction digestion analysis and DNA sequencing confirmed successful ligation of gene insert. The new FbpC expression vector was named pET28a FbpC Truncation.

### 3.3 Protein Expression

#### 3.3.1 Expression of FbpA B. cenocepacia.

pET28a FbpA B. cenocepacia J2315 was used to transform BL21 (DE3) competent cells before a single colony was used to inoculate 100ml kanamycin LB over night cultures and they were incubated overnight at 37°C. The overnight culture of cells was back diluted into 3L of 2YT media and cultured until an O.D$_{600}$ of 0.6 was met. At this point, 0.5mM IPTG was used to induce protein expression and the cells were incubated at 30°C for 3 hours. Pellets were collected by centrifugation and stored at -20°C.

#### 3.3.2 Expression of FbpA N. gonorrhoeae.

pTrc99 FbpA N. gonorrhoeae was transformed into Invitrogen One Shot® TOP10 E. coli competent cells. Single colonies were used to inoculate 1L 2YT Ampicillin media and the cells were incubated at 37°C overnight. Cells were centrifuged in the morning and stored in the freezer until further use.

#### 3.3.3 Expression of FbpC N. gonorrhoeae.

pET28a FbpC N. gonorrhoeae was transformed into either BL21 De3, BL21 Gold or C43 E. coli competent cells and selected for on Kanamycin (30mg/mL) LB agar. Overnight cultures in M9 minimal media, which were cultured at 37°C, were used to back dilute 3L Terrific Broth (TB) and the cells cultured until an O.D$_{600}$ of 0.8 – 1. Protein expression was induced with 0.1mM – 0.5 mM IPTG and cultures were incubated at 25°C for 3-18 hours (again see results and discussion).
3.3.4 Expression of FbpC B. cenocepacia

pET28a FbpC B. cenocepacia was transformed into BL21(DE3) chemically competent E. coli and selected for on kanamycin LB agar. A 5ml TB over day culture was used to inoculate 1L TB media, which was allowed to grow until an O.D_{600} of 1.0 was reached. At this point, 0.4mM IPTG was added to the culture, the temperature was lowered to 25°C and the cells were left to grow overnight. The cells were pelleted by centrifugation and tested for expression.

3.3.5 Expression of FbpC N. gonorrhoeae Δ111AA C-Terminal Truncation.

pET28a FbpC Truncation was used to transform E. coli BL21[DE3] competent cells before selection of transformants on LB Agar containing kanamycin antibiotic. A 20ml LB kanamycin liquid culture were inoculated with a single colony and incubated, shaking at 37°C overnight. The following morning, 4 x 100ml LB cultures were back diluted to O.D_{600} 0.1. Protein expression was induced when O.D_{600} 0.6 was reached using 0.1, 0.5, 1.0 and 2.5 mM IPTG and allowed to incubate at 30° for 3 hours. Cell pellets were collected by centrifugation.

3.4 Cell Lysis and Protein Purification.

3.4.1 CTAB Extraction of FbpA Proteins

The cell pellet was resuspended in CTAB extraction buffer consisting of 50mM HEPES pH 8.0, 1% cetyltrimethylammonium bromide (CTAB) and stirred at 37 °C for 3 hours. Cell free extract lysate was created by centrifugation at 25,000 g for 30 minutes at 4°C. CTAB was removed from the lysate by freeze/thaw followed by filtration or by dialysis at room temperature against 50 mM HEPES pH 6.8 (not necessary for purification by Fractogel).

3.4.2 Cation Ion Exchange Chromatography (IEX) Purification of FbpA B. cenocepacia.
Cation IEX Chromatography was performed using a HiPrep 16/10 SP FF Strong cation exchanger (GEHealthcare) equilibrated with 50mM HEPES pH 6.8. Lysate was .22µM filtered prior to injection onto the column and the protein was eluted using a 0-1M NaCl gradient, which was monitored at both 280 nm and 420 nm.

3.4.3 Batch Cation Ion Exchange Chromatography (IEX) Purification of FbpA N. gonorrhoeae.

FbpA *N. gonorrhoeae* was purified by batch method using Fractogel EMD SO₃⁻ chromatography media (Merck Millipore). 5 mL of Fractogel resin was repeatedly washed in a resuspension buffer consisting of 50 mM HEPES pH 6.8 and then added to cell free extract that was made as outlined in the above section. This resin/ cell free extract mix was stirred at 4°C for 1 hour. During this time a 5ml polypropylene column (Qiagen) was prepared by addition of a filter and the resin/ cell free extract mix was then decanted into the column. A stepped elution gradient of 50 mM HEPES pH 6.8 20 mM NaCl (20 column volume wash) 50mM NaCl, 100mM NaCl and 500 mM NaCl was then applied to the column and all flow through fractions were collected. Fractions were assessed for homogenous purification by SDS PAGE and could also be detected by eye due to a distinct red colour that is characteristic of the *holo* protein.

3.4.4 Analytical Size Exclusion Chromatography (SEC) of FbpA *B. cenocepacia*

Analytical Size Exclusion Chromatography (SEC) was performed using a 24ml bed volume Superdex™ 200 10/300 GL column (GEHealthcare) equilibrated with 50mM HEPES pH 7.5. SEC column had been previously calibrated (Appendix 1) 0.1mg protein sample was loaded onto the column. Size of proteins was calibrated by elution volume against calibration curve.

3.4.5 Preparatory Scale Gel Filtration Chromatography of FbpA *B. cenocepacia*. 61
To prepare FbpA *B. cenocepacia* for gel filtration chromatography, ion exchange fractions containing the protein were concentrated by ultrafiltration to a volume of 5ml by ultrafiltration using a Sartorius VivaSpin 10,000 Da molecular weight cut off (MWCO) ultrafiltration device. Gel filtration was performed using a HiPrep Sephacryl S-200 HR gel filtration chromatography column (GEHealthcare) on an Äkta FPLC system. The column was equilibrated with 10 mM HEPES pH 7.5 and 5ml of protein sample was loaded on to the column. 5 mL fractions were collected and analysed for protein content by monitoring at wavelengths 280 nm and 420 nm.

3.4.6 Lysis by sonication of *E. coli* FbpC *N. gonorrhoeae* Cell Cultures.

Cell pellet was resuspended in 25mM HEPES pH 7.5, 300mM NaCl, 10% Glycerol, 1mM ATP with DNAse (5ml per gram of wet cell pellet). Lysis was then performed by sonication on ice for 15 cycles of 30 seconds sonication, 30 seconds rest. Cell free extract was prepared by centrifugation at 25,000 *g* for 30 minutes at 4 °C using a SS-34 rotor head.

3.4.7 Lysis by Cell Disruption of *E. coli* FbpC *N. gonorrhoeae* Cell Cultures.

Cell pellet was re-suspended in 50ml 25mM HEPES (pH 7.5) with 300 mM NaCl, 10% Glycerol and 1mM ATP before lysis via cell disruption. To ensure that the cell pellets were fully homogenously resuspended, the 50ml resuspension mixture was passed repeatedly through the nozzle of a 50ml syringe, and also homogenised using a glass cell homogeniser. The cell resuspension was then passed three times through a pressure cell disruptor at 25 k PSI before clarification via centrifugation at 25,000 *g* for 30 minutes at 4 °C using a SS-34 rotor head.

3.4.8 Lysis by Freeze-Thaw Cycling of *E. coli* FbpC *N. gonorrhoeae* Cell Cultures.

Cell pellets were subjected to 5 x freeze thaw cycles by allowing the pellet to defrost originally and then re-freezing the pellet at -20°C. Following this the cell pellet was treated as in 2.3.4 and lysed by sonication.
3.4.9 Immobilised Metal Affinity Chromatography (IMAC) Purification of FbpC \textit{N. gonorrhoeae}.

Clarified supernatant from lysis protocols was .45 μM filtered and injected onto a Ni-NTA HisTrap 1ml HP column (GEHealthcare) via Äkta FPLC (GEHealthcare). The column was pre-equilibrated with re-suspension buffer (as above) plus 20mM imidazole. Care was taken to perform all steps at 4°C and the Äkta FPLC system was located in a cold room. An elution gradient of 0-500mM imidazole, with a stepped phase at 60mM, was applied and fractions were collected and analysed by SDS PAGE. Fractions that contained protein bands corresponding to the known molecular weight of FbpC were concentrated by ultrafiltration using a centrifugal Sartorius VivaSpin 30,000 Da molecular weight cut off (MWCO) ultrafiltration device.

3.4.10 UV-Visible Spectrophotometry.

All UV Vis spectrums presented in this work were collected using a Varian Cary® 50 Bio Spectrometer. Samples were examined using either a quartz cuvette or BRAND™ UV Cuvettes with an optical window down to 230 nm.

3.4.11 Liquid-Chromatography Mass-Spectrometry.

Samples for LC-MS analysis were submitted to SIRCAMS (UoE) and analysis was carried out Dr David Clarke. An Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), equipped with a monolithic PS-DVB analytical column (Dionex Corporation), was used for LC-MS analysis. Protein samples were prepared at a concentration of 5 μM.

MS data was acquired on a Bruker 12 Tesla SolariX FT-ICR (Bruker Daltonics, Billerica, MA) and analysed using DataAnalysis (Bruker Daltonics) software.

Isotope distributions of specific charge states were predicted using IsotopePattern software (Bruker Daltonics) from theoretical empirical formulae.
3.4.12 Native Mass-Spectrometry

Protein samples were concentrated to 50μM and buffer exchanged into 50mM ammonium acetate. MS data was acquired on a Bruker 12 Tesla SolarisX FT-ICR (Bruker Daltonics, Billerica, MA) and analysed using DataAnalysis (Bruker Daltonics) software.

Isotope distributions of specific charge states were predicted using IsotopePattern software (Bruker Daltonics) from theoretical empirical formulae.

3.5 Optically Transparent Thin Layer Cell (OTTLE) Potentiometric Titrations.

All titrations were carried out at 25°C and at pH 7 unless otherwise stated. UV Visible spectroscopy was performed on a Cary 50 and the potentiostat was an Autolab PGSTAT10.

Buffer A

| 0.5M KCl, 10% Glycerol and 50mM HEPES pH 7.0 |

Buffer B

| 0.5M KCl, 50mM HEPES pH 7.0 |

Protein samples were prepared before experiments by dialysis into the glycerol containing Buffer A and concentration to ~ 400μM.

Samples were prepared anaerobically in a Belle Technology glove box under a nitrogen atmosphere, with O₂ levels maintained at < 5ppm. Protein samples and buffers were introduced to the glove box at least 18 hours prior to use and allowed to de-gas.

Mediators were made up as a 5mg/mL stock and dissolved in Buffer A.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>E₀ (mV vs SHP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium ferricyanide</td>
<td>430</td>
</tr>
<tr>
<td>2,3,5,6-tetramethylphenylenediamine (DAD)</td>
<td>260</td>
</tr>
<tr>
<td>1,2-napthoquinione (12NQ)</td>
<td>135</td>
</tr>
<tr>
<td>N-methyl-1-hydroxyphenazonium methosulfate (PYO)</td>
<td>80</td>
</tr>
<tr>
<td>N-ethyl phenazonium ethosulfate (PES)</td>
<td>55</td>
</tr>
<tr>
<td>5-hydroxy-1,4-napthaquinone (5H14NQ)</td>
<td>-3</td>
</tr>
</tbody>
</table>
2-hydroxyl-1,4-napthaquinone (2H14NQ)  -50
Flavin mononucleotide (FMN)  -200
N’N’-dibenzyl-4,4-bipyridinium  -311
N,N’-dimethyl-4,4-bipyridinium  -430

The experiment was performed using a specially designed cell consisting of a modified quartz cuvette with a path length of 0.3mm. Electrodes used were a Pt/Rh (95/5) gauze working electrode (Englehardt, UK), a platinum wire counter electrode and an Ag/AgCl reference electrode (model MF2052 Bioanalytical Systems, IN, USA). Samples were injected into the OTTLE cell by using a Hamilton luer lock syringe with special care paid to ensure no bubbles were trapped in the sample. Buffer B was carefully layered on top with no mixing between the two layers. The OTTLE cell was fully assembled with the three electrodes and sealed before removal from the glove box.

A Cary 50 Probe UV Vis spectrometer was adapted to hold the quartz OTTLE cell and used to observe changes in UV spectra. The OTTLE cell was connected to the three electrodes and potential was applied. At each potential the sample was allowed to reach equilibrium and spectra was recorded for each point.

Figure 14: OTTLE Cell. A schematic representation of the cell that was used in all OTTLE experiments.

The reference electrode was calibrated against indigotrisulfonic acid (E₀ = -70 mV, pH 7.0) and all applied potentials were corrected relative to the standard hydrogen electrode.
3.6 Materials and Methods: Human Beta Defensin 2.

3.6.1 Construction of pET32b Thioredoxin-HBD2 fusion protein.

A synthetic 626bp HBD2 fusion protein with an N-terminal Thioredoxin tag and TEV protease site using *E. coli* optimised codons, and flanked with 5’ *XbaI* and 3’*HindIII* restriction sites was designed and purchased. pET32b was purchased from Novagen and used to construct the expression vector via standard recombinant techniques. 1 µL *XbaI* & 1 µL *HindIII* were used to digest 38 µL of both vector and insert plasmid in a double digest at 37°C for 4 hours with 5 µL NEB buffer 2 and 5 µL BSA. 1% agarose gel electrophoresis was used to examine for successful restriction and to separate restriction fragments prior to extraction with the QIAquick™ Gel Extraction Kit.

Vector and insert fragments were ligated for 5 minutes on ice according to the Roche Rapid Ligation Kit, before transformation into Top 10 competent cells. Colonies were cultured in 5ml LB Amp media, their plasmid DNA extracted and sequenced. This plasmid was named pET32b T.T.H. The expression vector was then used to transform BL21 (DE3) competent cells for protein expression.

3.6.2 pET32b Thioredoxin TEV HBD2 with an N-terminal His$_6$ tag.

A new gene incorporating an N-terminal His$_6$ was purchased from GenScript. Cloning of the plasmid was performed exactly as outlined previously. The new expression construct was named pET32b HTTHBD2

3.6.3 Expression of Trx-HBD2 fusions.

BL21 (DE3) pET32b T.T.H. cells were used to inoculate 2 x 250ml LB Amp cultures and incubated overnight at 37°C. These were then used to inoculate 5L of LB Amp to an O.D$_{600}$ of 0.1 and shaken at 37°C until an O.D$_{600}$ and protein expression was induced with 0.5mM IPTG. Expression was allowed to proceed for 3 hours before the pellet was harvested by centrifugation at 4,000 r.p.m for 20 minutes. The cell pellet was stored until further usage.
at -20 °C.

3.6.4 Purification of Trx.TEV.HBD2 & His.Trx.TEV.HBD2 fusion proteins

The cell pellet was defrosted and resuspended in 50ml resuspension buffer (50mM Tris-HCL pH 8.0, 300mM NaCl) containing a Complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche). The resuspension was then subjected to 10 x 30 seconds sonication and centrifuged at 10,000 r.p.m for 45 minutes at 4 °C. The supernatant was decanted, 45µM filtered (AKTA only) and kept at 4 °C. The pellet was resuspended 20ml of the above resuspension buffer, sonicated for 20 x 30 seconds and centrifuged for 45 minutes at 10,000 r.p.m 4 °C. The supernatant was again decanted, 45µM filtered and combined with the previous supernatant. The pellet was resuspended in SDS PAGE running buffer and both supernatant and pellet were examined for the presence of the fusion protein.

3.6.5 Ion Exchange Chromatography (IEX)

Solubility was established by the presence of Trx-HBD2 fusion protein in the supernatant, which was enriched via Ion Exchange Chromatography (IEX) on an AKTA system (GE Healthcare). The cell extract was loaded onto a HiLoad 26/10 Q Sepharose HP column which had been pre-equilibrated with 50 mM Tris pH 8.0 then elution was carried out with 50 mM Tris 0-1M NaCl gradient. 20 mL fractions were collected and assayed for protein presence via SDS PAGE analysis.

3.6.6 Cation Ion Exchange Chromatography using a Heparin column.

IEX was performed with a 1ml HiTrap Heparin HP column (GE Healthcare) according to manufacturer's instruction. To avoid protein precipitation and non-specific protein binding, the binding buffer already contained 100 mM NaCl. After equilibration of the column with binding buffer, the cleavage sample containing TEV-protease, cleaved HBD-2 and fusion partner Thioredoxin, was applied five times. Washing and elution fractions were applied and collected manually and analysed through SDS-PAGE.
3.6.7 Immobilised Metal Affinity Chromatography (IMAC)

3.6.7.1 Cobalt IMAC
Fractions containing the protein of interest were concentrated to a volume of 5ml using a 10,000 MWCO Spin filter (Sartorious). Fusion protein Trx-HBD2 was designed with a central His\textsubscript{6} tag and so was subjected to further purification via incubation for 2 hours with TALON Cobalt resin (ClonTech) equilibrated with 20mM Imidazole. A step-wise gradient of 50,100,200,300,400 & 500mM Imidazole was used to elute the protein and all fractions were again assayed for protein content by SDS PAGE.

3.6.7.2 Nickel IMAC
750 \(\mu\)L of nickel resin (Qiagen) equilibrated with resuspension buffer The resin was then incubated with the CFE at 4\(^\circ\)C for 1 h. Subsequently, samples were loaded onto single-use columns and batch eluted with increasing concentrations of imidazole. The different fractions (flow through (FT), 10 mL washing buffers with 20, 50, and 100 mM imidazole, 5 mL elution buffer) were collected using gravity filtration and analysed by 15\% SDS PAGE.

3.6.8 TEV Cleavage of Trx-HBD2.
Protein containing fractions were dialysed (BioDesign 8000 MWCO) in 3L TEV cleavage buffer (50 mM Tris, 10\% Glycerol, 300 mM NaCl, 0.5 mM EDTA pH 7.8) before the addition of 0.5 mM DTT and 0.4 mg (Stock 0.8 mg/mL) TEV protease followed by incubation at 30\(^\circ\)C for 4 hours. 4-12\% SDS PAGE (Invitrogen) analysis was performed to check for successful cleavage.

3.6.9 Purification of HBD2 by Nickel IMAC
1ml of NiNTA resin (Qiagen) was equilibrated in resuspension buffer and incubated for 1hr at 4\(^\circ\)C with the cleavage reaction mixture. Again samples were loaded onto a single use poly-propylene column and gravity filtration was used to collect the HBD2 containing flow through. A single elution step of 500mM imidazole was used, and all fractions were analysed by 4-12\% SDS PAGE.
3.7 Protein Chemistry

3.7.1 TCEP Reduction

10µM TCEP (Pierce) was added to protein samples and incubated at 25°C for 30 minutes in the dark.

3.7.2 NEM Alkylation

10µM NEM was added to protein samples and incubated, in the dark, for 30 minutes at 25°C. Samples were frozen until analysis.

3.7.3 Trypsin/ Trypsin & Chymotrypsin Digestion.

Trypsin (Bovine Pancreas, Sigma) and Chymotrypsin (Sigma) were used to enzymatically digest HBD2 peptide. The reaction was performed in 50mM Tris pH 7.8, 0.005% Triton X-100 and 20mM NaCl. Samples were incubated overnight at 37°C and the reaction was terminated by the addition of 0.2% formic acid.

3.8 Mass Spectrometry

3.8.1 Liquid Chromatography-Mass Spectrometry.

Samples for LC-MS analysis were submitted to SIRCAMS and analysis was carried out Dr David Clarke. An Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), equipped with a monolithic PS-DVB analytical column (Dionex Corporation), was used for LC-MS analysis. Samples containing about 1 µg of defensin were injected and washed with buffer A (2:98 acetonitrile:water, 0.05% formic acid) for 5 min, followed by a 20 min linear gradient elution (20 µl/min) into buffer B (80:20 acetonitrile:water, 0.05% formic acid). MS data was acquired on a Bruker 12 Tesla SolarisX FT-ICR (Bruker Daltonics, Billerica, MA) and analysed using DataAnalysis (Bruker Daltonics) software. Multiple charge states could
be observed this way.

Isotope distributions of specific charge states were predicted using IsotopePattern software (Bruker Daltonics) from theoretical empirical formulae. These were overlaid upon the recorded experimental data as scatter plots, with the theoretical apex of each isotope peak designated by a circle.
Chapter 4 Results and Discussion

4.1 Identification of a Putative Fe$^{3+}$ ABC Transporter from B. cenocepacia.

A BLASTp search using the FbpA N. gonorrhoeae amino acid sequence against the B. cenocepacia J2315 genome revealed a homologous open reading frame (ORF) with 24% sequence homology. This ORF, named BCAL1092, encodes a 37262 Da protein, putatively annotated as a periplasmic binding protein (82). Two further ORFs, BCAL 1091 & BCAL 1090 are located immediately downstream of BCAL1092 and result in a three gene operon consistent with other known ABC transporter complexes.

Figure 15: B. cenocepacia Fe$^{3+}$ ABC transporter is an operon consisting of three genes. BCAL refers to the gene nomenclature for chromosome 1 of B. cenocepacia. The flanking gene BCAL 1093 has not yet been annotated and BCAL 1089 is a transcription factor belonging to the AnsC family.

Periplasmic binding proteins are translated as immature pro-peptides with an N-terminal leader sequence that targets the peptide for translocation to the periplasm. Upon translocation the protein is folded, and the leader sequence cleaved, resulting in mature periplasmic protein. Periplasmic leader sequences contain conserved motifs, and analysis of the BCAL1092 protein using the program Signal P 4.0 (83) predicted a 28 amino acid leader sequence at the N-terminal of the encoded protein. This would result in a post-translational loss of a peptide approximately 2780 Da in size yielding a mature periplasmic protein with a molecular weight of 34485 Da. The gene BCAL 1092 was therefore considered to encode a putative periplasmic protein. Further protein BLASTp analysis against other known iron binding periplasmic binding proteins revealed that BCAL 1092 shares 89% sequence homology with Fbp from B. pertussis and 46% with FbpA from M.
*haemolytica* and on this basis BCAL1092 was designated a putative ferric binding protein (Figure 13).
Figure 16: Multiple sequence alignment of BCAL1092 from B. cenocepacia (B. cep) and ferroic binding proteins from B. pertussis (B. per) and M. haemolytica (M. haem). BCAL1092 has 46% sequence identity with the M. haemolytica homologue and 8% with B. pertussis. Conserved cysteine residues are indicated in yellow, red coloured residues are involved in carbonate coordination (M. haemolytica and B. pertussis). Blue residues contribute directly to iron binding.

B. cep
---AAEVLQYITIGFKLQPLDAFTQVSVKNTVFVDGLLERQVAEGQAQSPADVLM
B. per
GSHMSDEVSLYITIGFKLQPLDAFKDSQKIKVNTVFVDGLLERQVAEGDQSPADVLM
M. haem
---ANEVLYSYQPYLIEPLMNKEKDQTGQVINIFAQGCDTVKQEGLESLPADVLL

B. cep
TVDVGNLLDVLGVTQVRKLDALDQAPNLRTGMDYALSLPRVLYVEKDLKVD
B. per
TVDIQLDVLGVTQVRKLDALDQAPNLRTGMDYALSLPRVLYVEKDLKLD
M. haem
TVDIQVMEIVNADLAQKSDKVEKNINPAQFRSDQMWFGLTTAVIVYTSKDRVGKL

B. cep
A-FRYEDLAFPNKKGVCIRSCQHENLTVAMAIHDGAEATEWTLRGKANLAKATG
B. per
S-FRYGDALFPNKKGVCIRSCQHENLTVAMAIHDGAEATEWTLRGKANLAKAG
M. haem
AGFDYLALFEYKKGVRSGKSNVSLFAAMIEHYGIEKTAFLGKANLARLPQG

B. cep
GDRDVARDILGDCVGIGANAVGHMKBAPGDSKWDGDAIKNVRPTAFANAGGTHV
B. per
GDRDVARDILGDCVGIGANAVGHMKBAPGDSKWDGDAIKNVRPTAFANAGGTHV
M. haem
GDRQVKAIEKGDCYISGKSWGKMLEDH---KQKSFAAAAI---FPSEHG-THK

M. haem
NISGVVLKAKHAPKLVKLELYLVSPEQAQALYQANYEVPRANVKLDFPVSAGFTLK
B. per
NISGAHAAHAPKLVKLVLELYLSEPAQALYQANYEVPRAVKDLAVAAGFPLKV
M. haem
NISGVVLKAKHAPKLVKLELYLSEGAQGLYALNHEYVPKEGFEPASAVGWLTKS

M. haem
DPLPLADIAKHQASQQLVDFKDGVDFND------319
B. per
DTPVAEIAKYRQASELVDVFSDKGFDN------323
M. haem
DTIKLEDIAKNNYEAAGKLVKDFDDSEKK320
A model of the protein product of BCAL1092 was created using the x-ray crystal structure of *apo* FbpA *B. pertussis* and the free online bioinformatics tool SWISS model (84) (Figure 17).

**Figure 17: SWISS Model of BCAL1092 based on x-ray crystal structure of FbpA *B. pertussis* (89% amino acid sequence identity).** A: SWISS Model of protein encoded by BCAL1092 and B: x-ray crystal structure of *apo* FbpA *B. pertussis* (PDB20WS).

Sequence analysis of BCAL1090 identified a 257 amino acid protein with a predicted molecular weight of 28117 Da. Sequence alignment with NBD proteins from *N. gonorrhoeae* FbpC, *E. coli* vitamin B₁₂ (BtuCD) and *A. fulgidus* Molybdate/tungstate (ModC) ABC transporters revealed regions of amino acid sequence homology recognised as established ATPase signature motifs- the Walker A & B and H-loop sequences (Figure 14). The LSSGQ ABC transporter signature motif is also present. BCAL1090 is 95 amino acids shorter than FbpC *N. gonorrhoeae* and does not appear to share the putative C-terminal regulatory domain. The presence of the universally conserved motifs mentioned suggests that the protein encoded by BCAL 1090 is an ATPase enzyme.

BCAL 1091 encodes a 59.6 kDa protein that pFAM database analysis identifies as an ABC transporter associated transmembrane permease. The operon consisting of BCAL 1092, 1091 & 1090 was therefore putatively assigned as a ferric ABC transporter system belonging to *Burkholderia cenocepacia* J2315.
Figure 18: Sequence alignment showing conserved motifs of ABC transporter nucleotide binding domains from FbpC (B. cep), FbpC (N. gonorrhoeae) (N. gon), BtuD from E. coli and ModC from A. fulgidus. Red is the Walker A/P-loop, Olive is the ABC signature motif, Pink indicates the Walker B motif and Green is the H-loop motif.

<table>
<thead>
<tr>
<th></th>
<th>B. cep</th>
<th>N. gon</th>
<th>ModC</th>
<th>BtuD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-MNLLELDLCLIAYDTPHRTVGDGLALPRGDICLIEASCGGKTLLVRALKFGEPV</td>
<td>MTAALISHLSKSFQN---TVLNDISLSLDPGEILFIIASASCGHTLSLRCLAGFEQ</td>
<td>---MFLKVAKRLGN---FRNVDFEMGR-DYCVLQPTGACSVEFELASIALIGAVK</td>
<td>---MFLKVAKRLGN---FRNVDFEMGR-DYCVLQPTGACSVEFELASIALIGAVK</td>
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<tr>
<td></td>
<td>RMGRIVLDGFVAPSLVDPERRRIGMMPQDYALFPHLSAADNVAFGLR--RLPKAERR</td>
<td>DSGEISLSGKTFKSNLPLPRAITTFGLPRTGCSVPHLTLYRIYGLNGKGGRTAQER</td>
<td>DRGIEVRLNGADITP---LPERRGIFGPFQDYALFPHLSVRRNYINAYGLR---NVERVERD</td>
<td>SATKLMHRYLSQ---QTTPFPMPVWHYLYHLQDKTR---T</td>
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<tr>
<td></td>
<td>LRVAEMLELVLADGLASGDAYPELSGQQLRVALRALAPS---ELLDDEPFSNL</td>
<td>QRIEAMLELTGISELARGYPHELSSCGGQRVALRALDP---ELLLDEPFSAL</td>
<td>RVRREAELGIAHLLDKPARLSGQQRVALRALVIQP---ELLLDEPFSAL</td>
<td>SATKLMHRYLSQ---QTTPFPMPVWHYLYHLQDKTR---T</td>
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<tr>
<td></td>
<td>DNDTRERLADLRDILKHTGHIALVTVHRQAEEAFAAIADIGVMKQGQRQALWDTFALHHL</td>
<td>DEQLRQIREDMAALRNGKSAVFSYHSREAIQYDRAIAVMKQGRILQTAQHELRYQ</td>
<td>DLKTVGMLEEIIFVQREDIVILTVHRILIALVAMLADEVAVMNLRIGKEKILKFSA</td>
<td>ELLNDVAGALALDGLGRSTNQLSGGQVRVRLAIIQTQAPQLQIIDPMNSL</td>
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<tr>
<td></td>
<td>PAPAFTFVADFVRR---DALADER---ARALARGR</td>
<td>PADLDAVLFIEGIVFALNAUNGDAOCRGLRPVQGAPAGTRGTLILRPEQSLHPHS</td>
<td>KNGVEAFLSAR---NLLLKVSKILD---</td>
<td>PNLQAYGMLNR---RLDIEGHRMLIST---</td>
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<tr>
<td></td>
<td>APVCSIHAVVLKRTKARKTEISLRAQVTLTINLPSAPLSDGSAVLHLDGPLFFGNTL</td>
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**:** indicates the Walker B motif.
4.2 Construction of a BCAL1092 Expression vector.

Attempts were made to amplify BCAL 1092 from genomic *B. cenocepacia* J2315 DNA, but due to high GC% content this was not successful. Instead a 1052bp *E. coli* codon optimised synthetic gene was designed and purchased from GenScript (USA) as an insert in a pUC57 vector. This was used to transform DH5α *E. coli* competent cells and selected for on ampicillin LB Agar. Small-scale 5ml overnight LB cultures were sacrificed in order to isolate pUC57 BCAL1092 plasmid DNA. This was digested with *Ncol* and *XhoI* restriction enzymes and the presence of a ~1000bp insert was confirmed by 1% agarose gel electrophoresis. The DNA fragment was extracted from the gel and ligated into a kanamycin resistant pET28a vector that had been previously restricted with *Ncol* and *XhoI*. The ligation product was transformed into NEB High Efficiency DH5α *E. coli* competent cells, colonies were picked, cultured, and plasmid DNA isolated from them. Successful ligations were confirmed by restriction digestion and Sanger di-deoxy sequencing of the plasmid DNA. The expression vector was named pET28a FbpA J2315.

![Figure 19: Plasmid map of pET28a FbpA J2315 expression vector](Vector NTI® Advance Software). Plasmid contains a kanamycin selection marker.
4.3 Expression and Purification of Recombinant BCAL1092 (Putative Ferric Binding Protein from *B. cenocepacia*).

pET28a FbpA J2315 was used to transform BL21 [DE3] competent cells. Transformants were selected for on LB/Kanamycin agar plates. A single colony was used to inoculate 250ml 2YT media and incubated overnight at 37°C. This culture was back diluted into 3L 2YT /Kanamycin media to an O.D$_{600}$ of 0.1. Protein expression was induced with 0.5mM IPTG when O.D$_{600}$ reached 0.6 and expression was allowed to proceed for 3 hours at 30°C. Whole cell extracts were examined by SDS PAGE to confirm successful expression of the protein (Figure 20).

![Figure 20: SDS PAGE analysis of whole cells after IPTG induced expression of recombinant BCAL1092. An overexpressed band is visible at ~ 37 kDa which corresponds to the mass of the immature pre-FbpA peptide molecular weight of 37262 Da.]

Cell lysis was performed by resuspension in a 1% Cetyl trimethylammonium bromide (CTAB), 50mM HEPES (pH 7.5) extraction buffer and was carried out by stirring the suspension at 37°C (preventing the precipitation of CTAB) for three hours. CTAB is a cationic detergent that was used to ensure total lysis of the cell, making sure that the periplasmic fraction was fully solubilised. Experimental evidence suggested that mechanical lysis by sonication was not totally effective in solubilising the periplasm, and CTAB offers a rapid and cheap method of remedying this. Lysis by sonication resulted in a visibly red insoluble pellet, and as FbpA *N. gonorrhoeae* is red to the eye this was an indication that the protein of interest was present in the pellet. When CTAB was used to lyse the cells, there was no visible red colour in the insoluble fraction. The purification protocol was based upon that used to extract *N. gonorrhoeae* FbpA from *E. coli* as outlined by Taboy (59).
SDS PAGE analysis was used to determine that the protein was successfully extracted (Figure 21) and indicated that the protein is present in the soluble fraction.

**Figure 21: CTAB Lysis of BL21 DE3 recombinant BCAL1092 expressing cells.** LMW: Low Molecular Weight Marker. Cell: Whole cell pellet before lysis. Pellet is the insoluble cell debris collected after CTAB lysis and centrifugation. CTAB extract: soluble supernatant collected after CTAB lysis and centrifugation.

Mature BCAL1092 protein has a predicted isoelectric point of 8.82, and so cation ion Exchange Chromatography (IEC) was selected to purify the protein. A strong cation exchange (SO₃⁻) column (GE Healthcare, HiPrep 16/10 SP FF) was equilibrated with 50mM HEPES pH 6.8 on an AKTA FPLC and a 0-1M NaCl gradient was used to elute the protein (Figure 22).

**Figure 22: Purification of recombinant BCAL1092.** 


B: SDS PAGE analysis of cation IEX fractions 5, 6, 7 & 8.

Ferric binding proteins possess characteristic spectral handles when in iron bound holo form that are the result of ligand charge metal transfer (LMCT) a second wavelength
corresponding to a predicted LMCT was also used to monitor protein elution. 420 nm was selected for this because BLAST searches against the amino acid sequence encoded by BCAL1092 revealed high sequence homology with a \( \text{Fe}^{3+} \) binding protein from the Gram negative \( B. \) pertussis that has an LMCT with a \( \lambda_{\text{max}} \) at 416 nm. Therefore monitoring at 420 nm (Äkta units in 10 nm steps) was performed as the \textit{holo} LMCT band of FbpA \( B. \) cenocepacia may be of the same wavelength (73). Fractions were analysed by both SDS PAGE (Figure 22).

SDS PAGE revealed a \( \sim37 \) kDa protein that corresponds to the predicted immature mass of the protein (37.2 kDa). The expected molecular weight of mature \textit{holo} form of FbpA \( B. \) cenocepacia is 34.5 kDa, and it is difficult to determine a difference by SDS PAGE. However, the evidence of an LMCT at 420 nm is indicative of mature \textit{holo} FbpA, as immature protein is not able to bind iron. It was therefore assumed that FbpA \( B. \) cenocepacia had expressed been translocated to the periplasm, before post translational cleavage of the N-terminal pre-peptide followed by successful folding allowing adoption of the iron bound \textit{holo} form.

**4.4 Characterisation of FbpA \( B. \) cenocepacia.**

4.4.1 Analytical Size Exclusion Chromatography (SEC) of FbpA \( B. \) cenocepacia.

To ascertain the quaternary structure of FbpA \( B. \) cenocepacia analytical SEC was used. Analytical SEC separates molecules on the basis of their molecular weight while preserving intermolecular protein interactions. Using a calibrated SEC it is possible to determine the molecular weight of a protein (within a 10% margin of error) allowing an approximation of the oligomeric state of the protein in question.

0.01mg of FbpA \( B. \) cenocepacia was loaded onto a calibrated Superdex™ 200 10/300 GL SEC column (S200) and eluted (Figure 23).
Figure 23: Analytical S200 Size Exclusion Chromatography of FbpA *B. cenocepacia*. Blue line is absorbance at 280 nm and the red line is the absorbance at 420 nm. Elution fractions and elution volume are given on the X-axis and are red and black respectively.

The protein eluted in a single peak at 15ml (fractions 8+9), which agreed with the expected elution profile of a monomeric, approximately 35 kDa protein (± 3 kDa) (See appendix for S200 calibration). The predicted molecular weight of mature FbpA *B. cenocepacia* is 34503 Da.

**4.4.2 UV-Vis Spectroscopy of FbpA *B. cenocepacia***

UV-Vis spectroscopy was used to perform a 200-800 nm scan of the purified *holo* FbpA *B. cenocepacia* and demonstrated a characteristic LMCT band at 420 nm that is similar to that of other ferric binding proteins. FbpA *B. cenocepacia* is therefore expressed and purified as a *holo* complex (Figure 24). The λ max of the recombinant FbpA *B. cenocepacia* LMCT spectral handle is 421 nm (Figure 24)
**Figure 24:** UV Visible spectrum of native holo FbpA *B. cenocepacia*. (A) 200-800 nm scan of FbpA *B. cenocepacia*, the typical protein peak at 280 nm is indicative of aromatic residues. (B) The LMCT spectral handle characteristic of FbpA *B. Cenocepacia* has a maximal absorbance at 421 nm.

The molar extinction co-efficient ($E_{280}$) of FbpA *B. cenocepacia* was calculated using Vector NTI ® (Invitrogen) and determined to be 37080 M$^{-1}$ cm$^{-1}$. Application of Beer-Lambert Law ($A = E.C.L$) using the determined molecular weight of the mature protein (34,503Da) allowed calculation of the concentration of FbpA. The concentration was determined as 0.5mg/mL (14.6µM) in a final volume of 8.2ml. This corresponded to a yield of 5mg per 1L bacterial culture. Whilst the initial purifications of this protein resulted in moderately high protein yield, the key cation exchange column used in the process was retired due to loss of performance and could not be replaced at the time. As such a change in cation exchange media was unfortunately required. Instead of the 20ml bed volume chromatography column described previously, 1ml HiTrap FF SO$_3^-$ (GEHealthcare) disposable columns had to be used instead. This resulted in variety in yield, often < 0.2mg protein from 1L of bacterial culture, and made the purification somewhat unreliable.

### 4.4.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

*ICP-OES Analysis was kindly performed in collaboration with Dr. Lorna Eades, School of Chemistry, University of Edinburgh.*

Inductively coupled plasma optical emission spectroscopy is an analytical technique that can be used to detect trace metals and some non-metallic elements (such as sulphur and phosphorus). The analyte is passed through argon plasma that excites
atoms causing emission of electromagnetic radiation that is then detected by emission spectroscopy. Calibration with known elements of interest (in this case iron) allows comparison of emission spectra between known standards and the sample being examined. This technique was used to assess if iron was present in purified, recombinant FbpA *B. cenocepacia* samples. Also, to determine if FbpA *B. cenocepacia* binds iron via a phosphate anion in a fashion analogous to FbpA *N. gonorrhoeae* analysis of phosphorus was also performed.

ICP-OES confirmed that iron is present in FbpA *B. cenocepacia*. Stochiometric analysis of iron to protein monomer gave a ratio of ~0.5 in triplicate samples. The expected stochiometry is 1:1 as in other FbpA proteins, but it is not an unreasonable assumption that half of the recombinant protein in the analysed sample may be in the apo form. Analytical size exclusion chromatography did not indicate that FbpA *B. cenocepacia* is a dimer in solution so it is unlikely that stoichiometry of iron binding is 2 protein to 1 iron. Therefore it cannot be said with certainty what the stoichiometry of iron to protein is, but can be said that FbpA *B. cenocepacia* is an iron binding protein. Phosphorus and sulphur were not detected in the "as purified sample "and therefore, unlike FbpA *N. gonorrhoeae*, recombinant FbpA *B. cenocepacia* does not utilise phosphate as a synergistic anion when purified from *E. coli*.

4.4.4 Mass spectrometry Analysis of FbpA *B.cenocepacia*.

All LC-FTIR-MS experiments were kindly performed with the help of Dr David Clarke of the SIRCAMS/Campopiano groups, School of Chemistry, University of Edinburgh.

Following purification, the mass of recombinant FbpA *B. cenocepacia* was determined by high resolution LC ESI FT-ICR MS under denaturing conditions (50:49:1 MeOH:H₂O:Formic Acid (FA)). FbpA *B. cenocepacia* produces a characteristic ion envelope of which the most abundant ion is the [M+33H]⁻33 charge state. Deconvolution of this major peak reveals an average mass of 34503.5 Da, which corresponds exactly with the theoretical average mass predicated by the amino acid sequence of mature FbpA *B. cenocepacia*. The the isotopic distribution distribution of the [M+33]³³⁺ charge state is indicative of FbpA containing a single disulfide bond (empirical formula C₁₅₃₇H₂₄₅₆N₄₃₄O₄₅₈S₅) (Figure 25). Disulfide bond formation is consistent with a presumed oxidative periplasmic environment that is the cellular location of the protein(75). Slight
differences in m/z observed between the spectrum and the theoretical distribution map are within the margin of error for this measurement.

![Diagram](image)

**Figure 25: ESI-FT-ICR Mass spectrometry of FbpA B. cenocepacia.** The deconvoluted average mass was determined to be 34503.5 Da. Comparison of the theoretical isotopic distribution spectrum pertaining to the empirical formula for FbpA containing a single disulfide bond (A), with the exact mass of the abundant [M+33]^{33+} charge state (B) confirms the predicted mass of FbpA + 1 disulfide bond.

### 4.4.5 Native Mass Spectrometry of FbpA B. cenocepacia

*All Native MS experiments were kindly performed with the help of Dr David Clarke of the SIRCAMS/Campopiano groups, School of Chemistry, University of Edinburgh.*

To gain more information about iron co-ordination by FbpA B. cenocepacia, native ESI-FT-ICR-MS was employed. Native mass spectrometry is a technique that allows analysis of protein structure by preservation of non-covalent interactions such as quaternary structure (85). This method of analysis will not denature a protein complex and so in principle the mass detected should be that of as purified *holo* FbpA plus the mass of ferric iron and possible synergistic anion still bound.
FbpA at a concentration of 50µM was buffer exchanged into 50 mM ammonium acetate and analysed by direct infusion ESI-FT-ICR MS. Native MS analysis of FbpA *B. cenocepacia* presents a spectrum with a dominant [M+12]^{12+} charge state that deconvolutes to an average mass of 34745 ±2 Da (Figure 26). This was a mass increase of 242.5 Da when compared to a sample that had been examined under denaturing conditions. As the exogenous synergistic anion of FbpA *N. gonorrhoeae* is known to be facile these two spectra were obtained from freshly purified holo recombinant protein (75).

![Figure 26](image)

**Figure 26:** (A) Typical ion envelope of FbpA *B. cenocepacia* under denaturing where the abundant charge state of [M+33H]^{33+} is visible. (B) ESI- FT-ICR MS of FbpA *B. cenocepacia* examined under native conditions. Deconvolution of the 13+ and 12+ charge state results in an average mass of 34745 ±2 Da.

Sequence alignments of FbpA with other structurally characterised ferric binding proteins revealed high homology with FbpA *B. pertussis* (75).
Figure 16). The x-ray crystal structure of this Fbp has been solved (PDB 2OWT) with carbonate as the synergistic anion. Given the high sequence homology, and the similar holo LMCT absorbance maximum of the two proteins it was assumed that FbpA B. cenocepacia would utilise the same synergistic anion as FbpA B. pertussis. However, the increase in mass of 242.5 Da is larger than would be observed for [FbpA-Fe\(^{3+}\)-CO\(_3\)] complex (115.91 Da) indicating that the 421 nm holo LMCT displayed by FbpA B. cenocepacia is not the result of a carbonate ligand.

It is worth noting at this point that FbpA B. pertussis was expressed as a cytoplasmic protein that lacked an N-terminal leader sequence (73). Due to this, the protein was purified in the apo form and was converted to holo by re-loading with ferrous sulphate + X (whereby X = carbonate/oxalate/citrate). Crystallographic data was obtained for two re-loaded proteins using either carbonate or two oxalate molecules (C\(_2\)O\(_4\)H\(_6\)) as synergistic anions. Both carbonate and oxalate bound structures had co-ordinated ferric iron with pseudo-octahedral geometry but was carbonate was dismissed as the “native” anion as the form of the protein was more open when compared to that of the carbonate binding FbpA. No crystals could be obtained for citrate bound complex. Competition assays, where UV-Vis spectroscopy was used to monitor changes in LMCT absorbance in the presence of an excess of chelator, supported carbonate as the native ligand and so FbpA B. pertussis was designated as a carbonate binding protein. As B. pertussis was not purified as a holo complex it maybe that designation as a carbonate binding protein is incorrect.

Ferric binding proteins have been documented as capable of utilising other physiologically available anions including phosphate, arsenate, sulphate, oxalate and citrate (75). Of these, citrate and oxalate are of a similar mass to that of the unidentified ligand measured by native MS and in combination with Fe\(^{3+}\) have an average mass of 243.95 Da and 232 Da respectively. These two average masses are close, but it is possible to discern between the two by mapping the theoretical isotopic distribution of each and overlaying that map with the native spectrum of FbpA. The instrument used to perform this experiment (Bruker 12T SolariX FT-ICR MS) has the resolving power to do this.
As a control experiment, FbpA was converted to apo by dialysis against a vast excess of the chelator tri-sodium citrate. Conversion to apo was observed by UV-Visible spectroscopy and is concomitant with a loss of the LMCT band at 421 nm.

_Holo_ “as purified” and _apo_ samples were then dialysed into ammonium acetate buffer (native MS compatible) and analysed under identical conditions. The _apo_ sample mass spectrum displayed three prominent species consistent with the mass of _apo_ FbpA _B. cenocepacia_, [FbpA + Fe$^{3+}$] and [FbpA + Fe$^{3+}$ + Anion X]. _Apo_ FbpA was not detectable in the “as purified” sample that had not been treated with citrate as a chelator molecule (Figure 27).

**Figure 27:** Native ESI-MS of FbpA _B. cenocepacia_. (A) ESI FT-ICR MS spectrum of the 13$^+$ charge state of FbpA _B. cenocepacia_ dialysed against an excess of the chelator tri-sodium citrate (50mM) and (B) of the "as
purified" native holo FbpA B. cenocepacia (purified in HEPES pH 6.8, dialysed into 50mM Ammonium acetate for analysis). * Indicates undefined.

To compare a theoretical isotopic distribution map for each possible synergistic anion, the empirical formula for each holo complex had to be calculated. Isotope distribution maps were created using Bruker Daltonics Software.

Isotopic mapping of the [M+13]+13 charge state compared with theoretical isotopic distribution of apo FbpA B. cenocepacia, [FbpA + Fe3+] and [FbpA+Fe3++ citrate/NTA/Oxalate/CO3−] strongly suggested that citrate is the synergistic anion that recombinant FbpA B. cenocepacia utilises to co-ordinate Fe3+ in E. coli (Figure 28).

Interestingly, in the same study that solved the structure of FbpA B. pertussis, a re-loaded FbpA iron citrate complex was isolated and absorbance of an LMCT band with a maximum 428 nm was observed in the UV spectrum (73). This is comparable to the maximum LMCT band of holo FbpA B. cenocepacia that is visible at 421 nm and supports the hypothesis that citrate may be the synergistic anion in this complex.

Although there are other examples of FbpA proteins that can bind citrate, this is the first example of a recombinant FbpA protein that has been purified with in the ferric citrate bound form, suggesting that this is the natural synergistic anion for this protein.
Figure 28: Native ESI-MS of FbpA *B. cenocepacia*. Isotopic distribution of (A) Apo-FbpA, (B) FbpA-[Fe$^{3+}$] and (C) FbpA-[Fe$^{3+}$ Citrate]. Red circle = Theoretical fit calculate from empirical formula for each species of FbpA which has been laid over the actual spectrum collected from Native ESI-MS. Green circle = theoretical fit calculated from the empirical formula of FbpA-[Fe$^{3+}$ 2 x oxalate].
4.4.6 Discussion

The chemistry of aqueous ferric citrate in solution is extremely complex as a number of multinuclear metal ligand complexes can form that are in dynamic equilibrium. A number of small molecule studies have attempted to deconvolute speciation of ferric citrate in solution, resulting in the structural characterisation of five different anionic complexes from aqueous solution - ferric monocitrate, ferric dicitrate, diferric dicitrate and triferric-tricitrate (Figure 29) (86). Factors that can affect the composition of ferric citrate complexes include molar ratio, the iron salt available as well as pH and temperature and as such the composition of ferric citrate available in the periplasm is currently unknown.

![Figure 29: Examples of ferric citrate species bound to protein and in solution. (A) Representation of a protein bound ferric iron atom. In this model, citrate is acting as an exogenous anion and contributes three oxygen ligands toward complete co-ordination of the ferric iron. Three tyrosine residues complete the co-ordination sphere. (B) Structure of ferric dicitrate illustrated in pymol out to clearly represent the six contributing oxygen ligands provided by the two citrate molecules and (C) as illustrated in Chemdraw.](image)

As ferric binding proteins are known to co-ordinate iron via a conserved di/tri-tyrosinal motif then FbpA *B. cenocepacia* may contribute three ligands via amino acid side chains and complete octahedral co-ordination of the iron atom via citrate.

As of yet, only the ferric citrate transporting FecABCDE system of *E. coli* and the *Bacillus cereus* ferric citrate binding protein FctC have been biochemically characterised. FecABCDE is a TonB dependant transporter system that is regulated by the global iron uptake regulator Fur. An OM receptor (FecA) transports Fe$_2$Cit$_2$ through to the periplasm where it is hypothetically complexed by the periplasmic binding protein FecB. Transport to the cytoplasm is completed by the transmembrane
permeases FecC/D and ATPase FecE via a Fur-regulated TonB dependant ABC transporter system (87). The only part of this complex to be experimentally characterised is FecA, and the additional components have been identified by in silico bioinformatic analysis of the FecA containing operon.

In the Gram positive organism *B. cereus*, FctC has been identified as a ferric citrate binding protein that binds Fe$_2$Cit$_2$ or Fe$_3$Cit$_3$ with a selective preference for the latter. Interestingly, FctC was shown not to bind ferric mono-citrate, indicating that there is preference amongst ferric citrate binding proteins even for ferric citrate speciation (88). A *B. cereus* strain attenuated in ferric citrate transport was not growth defective when cultured in iron limited conditions, but was defective when ferric citrate was provided as the sole iron source. From this it can be concluded that at least two iron import systems are present in *B. cereus*. Further to this, the attenuated strain showed limited virulence in a lepidopteran infection model and therefore ferric citrate transport was shown to be essential for full virulence of *B. cereus* (89).

The Gram positive *B. subtilis* YfmC protein has also been identified as a ferric citrate binding protein belonging to the Fur-regulated YfmCDEF Fe-citrate transporter complex. Whilst this system has not been biochemically characterised, it was demonstrated that YfmCDEF was required for optimal growth when citrate was provided as the sole carbon source (90). *Burkholderia* species have been documented as able to utilise citrate as a sole carbon source but the pathway that confers this characteristic has not been defined (8).

We wanted to investigate if FbpA *B. cenocepacia* shared any similarity with the proteins outlined above and so BLASTp searches against the *B. cenocepacia* J2315 genome using the amino acid sequences of FecB, FctC and YfmC were performed. It was expected that BCAL1092 would be a match, however the searched identified only a gene named *OrbB* as a hit with any significance > 20% and BCAL1092 was not listed in the search results. *OrbB* belongs to a gene cluster involved in the biosynthesis of the siderophore ornibactin and is annotated as a periplasmic binding component of an ABC transporter (91). Based on bioinformatics analysis of known ferric citrate systems FbpA *B. cenocepacia* does not share any significant homology with other ferric transport systems currently described and so if FbpA *B. cenocepacia* is a ferric citrate transporter then it is likely that *B. cenocepacia* also has at least two iron transport systems (ornibactin siderophore and ferric citrate) in a fashion analogous to *B. cereus*.
The species *Burkholderia* are documented as capable of utilising citrate as a sole carbon source and genes annotated as involved in citrate catabolism are up-regulated in clinical isolates of *B. cenocepacia* J2315. In the same analysis it was discovered that BCAL1092 was also up-regulated in clinical strains and so were the ornibactin synthesis genes *OrbJ* and *OrbK*, although not *OrbB* (BCAL1694)(92-93). As *Burkholderia* species are able to colonise plants, and plants are known to utilise ferric citrate as an iron storage system in xylem sap, the bacteria may have evolved a specific ferric citrate homostatic mechanism (94). Recently, an FbpA homologue from the marine organism *Marinobacter algicola* has been reported as using borate as a synergistic anion. In the marine environment borate is present in high concentration compared to the non-marine world and so the use of this element as a synergistic anion is another example of possible adaptation to environment through iron sequestration pathways (95). Based on this evidence it may be that FbpA *B. cenocepacia* is in fact part of a ferric citrate uptake system that is functional in iron transport and citrate catabolism.
4.5 Crystallisation of holo FbpA B. cenocepacia.

X-ray crystallography experiments were set up in collaboration with Dr. Simon Newstead, Department of Biochemistry, University of Oxford.

FbpA B. cenocepacia was freshly purified in HEPES buffer and concentrated to 400uM, and snap frozen for transport to Oxford for crystallisation trails.

Crystallisation conditions for holo FbpA B. cenocepacia were screened using a commercially available PACT screen from Molecular Dimensions®. In the original screen, crystals were obtained in conditions that contained 20% PEG 6000, 0.1M Tris pH 8.0 and 2 mM ZnCl₂ but these resulted in poor diffraction resolution (> 3 Å). Therefore further optimisation work was undertaken and crystallisation conditions for holo FbpA B. cenocepacia were obtained using: 16% PEG 6000, 0.1M Tris pH 8.0, 14 mM ZnCl₂. This screen was optimised from an original 'hit' in the commercially available screen. This new crystal of holo FbpA B. cenocepacia was brown/orange colour to the eye and diffracted to 1.7Å resolution.

Primary modelling indicates that a single Fe atom is bound to the oxygen atom of tyrosine 199. The characteristic tyrosine binding motif of FbpA proteins is further represented by tyrosine residues 200 and 143 that are swung toward the iron atom with respective oxygen atoms positioned 2.2 Å and 2 Å distance. A mass of density is visible in close proximity to the iron atom and occupies the space a synergistic anion may be located. As of yet the identity of this density is unknown but it is consistent with a molecule that possesses 4 atoms arranged in a planar confirmation. The three atoms at the base of the molecule are positioned toward the iron atom with the central atom located at 1.8 Å distance and the two outer atoms are 2.3 Å and 2.6 Å. The density is not consistent with that of citrate, nor does it correlate with an oxalate molecule and therefore does not support the finding suggested by native mass spectrometry studies. However, further refinement work is required to determine the mass identified but possible candidates are carbonate, glycerol (used as a cryo-protectant when storing the protein) or four waters of which three are oriented toward the Fe centre (Figure 30).
Figure 30: View of the iron binding site of FbpA *B. cenocepacia*. The three tyrosine residues implicated in iron binding are green and Tyr199 is illustrated as bound to the central iron atom (A). The 4 atom, planar configuration of the unknown ligand caught in the structure is visible as purple spheres (B) where the base of atom is angled toward the iron atom.

At present it appears that the iron bound structure was not caught in complete *holo* form as the iron is not completely co-ordinated with pseudo-octahedral geometry. The supposed water molecules in the active site suggest that citrate was not tightly bound and came out during crystallisation. It should be noted that the protein sample used in the crystallisation process had been subjected to several free/thaw cycles and so maybe not be representative of freshly purified FbpA *B. cenocepacia* that was used in Native MS experiments. To further investigate this, the crystal has been harvested and sent to SIRCAMS (UoE) to attempt to obtain a native mass spectrum from the crystal.

Currently, further crystal trails are being carried out with the hope of successfully determining the synergistic anion of FbpA *B. cenocepacia*. 
4.6 Optically Transparent Thin Layer Cell (OTTLE) Potentiometric Titrations.

The transport of iron across biological membranes is of significant importance whether referring to eukaryotic or prokaryotic cells. In Gram negative bacteria, iron acquisition is considered a virulence factor and crucial for pathogenesis of a human host. The pathways by which gram negative bacteria, and in particular the obligate human pathogen *N. gonorrhoeae*, transport iron have been the subject of considerable research, but the mechanism by which iron sequestering proteins release their cargo upon interaction with a cognate receptor has not yet been determined.

FbpA *N. gonorrhoeae* has a $K_d$ for Fe$^{3+}$ of $4 \times 10^{-18}$ M (59). This binding affinity is exceptionally strong and reflects the scarcity of free iron within the environment. Upon reduction of Fe$^{3+}$ to Fe$^{2+}$ the binding affinity of FbpA *N. gonorrhoeae* for the ferrous ion is reduced by an estimated 14 orders of magnitude implying that reduction may be an important factor in transferal of iron from ferric binding protein to permease (75). The midpoint potential of recombinant FbpA *N. gonorrhoeae* has been measured using OTTLE potentiometric titrations resulting in a midpoint potential ($E_M$) value of -305 mV (59). The FbpA sample examined utilised phosphate as the synergistic anion and all experiments were performed at pH 6.5, to reflect the expected pH of the periplasm. At this redox potential, *holo* FbpA *N. gonorrhoeae* is in the range of physiologically available NADH/NADPH driven reductases (95).

A later study focused on the effect of the synergistic anion upon redox potential using a range of physiologically available anions. *Apo*-FbpA *N. gonorrhoeae* was incubated with various anions and the midpoint potential of these re-loaded [FbpA-Fe-X] complexes determined by OTTLE titrations. The range of midpoint potentials displayed by the [FbpA+Fe+X] complexes varied from -300 mV (PO$_4^-$) to -184 mV (NTA) presenting an oxidative shift of 116 mV dependant on synergistic anion. The effective binding constant of [FbpA + PO$_4^{3-}$] for Fe$^{3+}$ was determined as $4.2 \times 10^{18}$ M$^{-1}$ and was shown to decrease to $1.4 \times 10^{17}$ M$^{-1}$ when in complex with other anions. Interchange of the synergistic anion therefore appears to have a significant effect on the reduction potential of FbpA *N. gonorrhoeae* but does not dramatically alter the binding affinity of FbpA *N. gonorrhoeae* for ferric iron.

We wanted to investigate the electrochemical properties of FbpA *B. cenocepacia*, by measuring the midpoint potential of this novel FbpA. Specifically the idea that
reduction potential may play a role in the release of iron from the periplasm to the ABC transporter complex has not been addressed in other FbpA characterisation studies and so determining the midpoint potential of other FbpA proteins may contribute to understanding the iron release mechanism.

4.6.1 OTTLE Potentiometric Titrations of FbpA *N. gonorrhoeae*.

To provide a control for the OTTLE titration the midpoint potential of FbpA *N. gonorrhoeae* [FbpA+ Fe + PO₄] was measured. This experiment has literature precedent, allowing a degree of comparison between our experimental results and cited literature values. Experimental conditions were set up to mirror those used by Taboy et al (59).

Briefly, FbpA *N. gonorrhoea* and FbpA *B. cenocepacia* were concentrated to 400uM in 0.5ml and an excess of the electron mediator methyl viologen (Eₒ = -420 mV) was added to each sample. The sample was stored for 24 hours under nitrogen to remove oxygen. All buffers used were degassed and the OTTLE cell was assembled anaerobically in a glove box. Once sealed, the OTTLE cell was placed in a UV Vis spectrometer and connected to a potentiostat. The OTTLE electrode was calibrated against indigotrisulfonic acid and all reaction measurements have been corrected against this (+203 mV). As literature values place the midpoint potential of FbpA *N. gonorrhoeae* as -290 mV a starting potential of 0 mV was applied for 30 minutes to assess the stability of the protein sample under an electrical current. Following this the applied potential was reduced by 20 mV and monitored by potentiostat and UV Vis spectroscopy until equilibrium was reached (Figure 31). Reduction of holo FbpA is concomitant with loss of FbpA *N. gonorrhoeae* LMCT at 481 nm and so direct measurement can be performed using UV Vis spectroscopy. Once full loss of absorption at 481 nm was observed the potential titration was reversed, and increasingly oxidative potentials were applied in 20 mV increments.

The measured midpoint potential of FbpA *N. gonorrhoeae* was -489 mV, corrected by reference electrode calibration to -286 mV (Figure 31). This is an oxidative shift of 4 mV compared to the published values for FbpA *N. gonorrhoeae* and is within an acceptable margin of error. However it should be noted that unlike the results stated in Taboy et al, equilibrium at each potential was not achieved within 30 minutes. In fact, each OTTLE experiment ran for at least 36 hours, and as can be observed in the Nernst
plot for this data, there was a single reduction step indicating that once reduction began equilibrium was not reached until the entire sample was reduced. Furthermore, we were unable to oxidise the protein to *holo* form by oxidising potential titrations. Taboy was not able to demonstrate the reverse titration reaction in the OTTLE cell, but stated that after removing the sample from the OTTLE cell and exposing it to air, *holo* FbpA was detected (a red colour that is visible to the eye). Despite monitoring at 481 nm for up to 24 hours, no regeneration of the LMCT band was detected in these experiments.

As the measured reduction potential of FbpA *N. gonorrhoeae* was repeatedly close to the cited literature value, we decided to attempt to measure the midpoint potential of FbpA *B. cenocepacia* under identical conditions. In this way, a direct comparison could be made between the two proteins.

### 4.6.2 OTTLE Potentiometric Titrations of FbpA *B. cenocepacia*.

Initial experimental designs were based on the determined electrochemical characteristics of FbpA *N. gonorrhoeae* but some optimisation was required. The addition of mediators to effectively shuttle and disperse electrons from the electrode interface across the concentrated protein sample is essential in an OTTLE experiment. Considerations when choosing a mediator include the reduction potential \( E_0 \) as it must be relatively close to the sample being measured and there should be no UV crossover between mediator and sample that can distort experimental readings.

The reduction half potential requirement for a mediator can be estimated by the following equation:

\[
|E^0_M - E^0_P| \leq \frac{2RT}{nF_i}
\]

Mediators should also not interact with the sample in anyway (96). Several molecules, including anthraquinone 2-6 disulfonic acid (ANS, \( E_0 = 185 \text{ mV} \)), anthraquinone 2-sulfonic acid (AQS, \( E_0 = -225 \text{ mV} \)), benzyl viologen (\( E_0 = -311 \text{ mV} \)) and methyl viologen (\( E_0 = -420 \text{ mV} \)) were investigated as appropriate mediators but only benzyl and methyl viologen were deemed suitable.
OTTLE experiments using FbpA *B. cenocepacia* were assembled as outlined previously and repeated in triplicate. The measured $E_{\text{RXN}}$ was -346 mV, corrected to $E_M = -143$ mV against a calibrated reference electrode (Figure 32). As observed in our replication of the FbpA *N. gonorrhoeae* potentiometric titration, the reduction of FbpA *B. cenocepacia* was not reversible and the sample could not be restored to an oxidised *holo* state. As in the previous experiment, once reduction of the protein sample had begun, equilibrium was only observed after total reduction of the sample as monitored by UV Vis spectroscopy. Reaching equilibrium took several hours, with all OTTLE experiments taking over 48 hours to complete. Protein stability was monitored by absorbance at 280 nm and precipitate formation was only significant after 72+ hours. Slow equilibrium can be explained by the midpoint potential of benzyl viologen (-311 mV) being too reductive compared to the measured midpoint potential of the protein. In effect, the mediator will not have been an effective electron shuttle at this potential, resulting in a very slow equilibrium. However, other mediators (ANS, AQS) appeared to shift the midpoint potential of FbpA *N. gonorrhoeae* in control experiments and so were avoided (ANS shifted the measured $E_M$ to -148 mV).

There is a significant change in protein binding kinetics upon reduction of Fe$^{3+}$ to Fe$^{2+}$ that is illustrated in FbpA proteins and the human iron binding protein transferrin. The $K_d$ of FbpA *N. gonorrhoeae* and transferrin is significantly lower upon reduction of Fe$^{3+}$ to Fe$^{2+}$ and so reduction of iron is followed by an equilibrium coupled dissociation of iron from the protein (Equation 2+3).

\[
\text{Equation 2: } FbpA \text{Fe}^{3+} + e^- \rightleftharpoons FbpA \text{Fe}^{2+}
\]

\[
\text{Equation 3: } FbpA \text{Fe}^{2+} \rightleftharpoons \text{Fe}^{2+}_{\text{aq}} + FbpA
\]

As discussed by Taboy et al, the result of this is a non-Nernstian electrochemical response and as the proportion of [Ox]/[Red] species is used to calculate the Nernst plot, the experimentally measured concentration of reduced FbpA must be corrected for the dissociation constant ($K_d$) of FbpA Fe$^{2+}$ (75).
Taking into account the affinity of apo FbpA for Fe$^{2+}$ (estimated $K_d = 10^{-3}$ M based on transferrin $K_d$) the reduction potential of FbpA *N. gonorrhoeae* is corrected by Taboy to -305 mV (76).

The terms defined this process require the concentrations of each species at the point of equilibrium to be known - but during the reduction of FbpA *B. cenocepacia* equilibrium was not reached until reduction was complete (this was also observed in FbpA *N. gonorrhoeae* control experiments). Therefore the absolute validity of the measured value for FbpA *B. cenocepacia* cannot be corrected as in the manner previously outlined as the reaction was never in equilibrium.

The accuracy of measurement experimental results can be called into question by the systematic problems associated with using a Nernstian model to attempt to measure a non-Nernstian response. However, replicating the established OTTLE FbpA *N. gonorrhoeae* experiment provided consistently reproducible data and so a comparison between FbpA *N. gonorrhoeae* and FbpA *B. cenocepacia* experimental data can still be considered valid. Under identical experimental conditions the measured midpoint potential of recombinant FbpA *B. cenocepacia* is -346 mV - 138 mV oxidative in comparison to FbpA *N. gonorrhoeae* measured at -484 mV.
Figure 31: UV Visible spectra of FbpA *N. gonorrhoeae* potentiometric titration (A & B) and Nernst plot (C). λ max of FbpA *N. gonorrhoeae* LMCT = 481 nm. The shoulder appearing at 400 nm and 530 nm is reduced methyl viologen.
Figure 32: OTTLE Potentiometric titration of FbpA *B. cenocepacia*. UV Visible spectra of the experiment (A & B) and Nernst plot of the data obtained (C). \( \lambda_{\text{max}} \) of FbpA *B. cenocepacia* LMCT = 421 nm.
4.6.3 Discussion

Electrochemical analysis of FbpA B. cenocepacia by OTTLE potentiometric analysis was performed in an attempt to further understand the mechanism of iron release from FbpA. The measured midpoint potential of FbpA B. cenocepacia was -143 mV compared to FbpA N. gonorrhoeae at -286 mV. Facile anion exchange has been observed in FbpA N. gonorrhoeae and it has been proposed that FbpA functions not only to control movement of iron through the periplasmic space, but also to keep a pool of buffered ferric iron available for delivery to the cytoplasm upon demand (55). Based on spectroelectrochemical analysis of FbpA N. gonorrhoeae, anion exchange of phosphate to citrate results in a 138 mV oxidative shift that decreases the energy requirement for reductive release of iron. It has therefore been proposed that anion composition of the periplasm can affect synergistic anion binding and influence the reduction potential required for transfer of iron to the cytoplasm. In this model, exchange of phosphate for citrate would occur prior to receptor interaction, lowering the energy requirement for reductive release of iron and therefore making it more favourable. Stability constants for phosphate and citrate complexes have been determined previously and shown to decrease from 4.2x10^{-18} M to 1.4x10^{-17} M, demonstrating that anion exchange does not significantly de-stabilise the [FbpA+Fe^{3+}+X] complex.

Both FbpA N. gonorrhoeae and FbpA B. cenocepacia were purified as recombinant holo form from the periplasm of an E. coli BL21 [DE3] strain in which neither of their respective transporter components were expressed. As the culture media, pH and purification buffers are identical, an argument can be made that the periplasmic environment both proteins were expressed in is highly similar, if not identical. If periplasmic environment modulates anion selection with an aim to control reduction potential it could be predicted that both proteins would utilise a synergistic anion that results in a similar midpoint potential when purified under identical conditions. The results in this chapter indicate that this is not the case and highlight that building a model for iron release based around the electrochemical analysis of a single characterised member of a protein class (as is currently happening) as un-reliable.
4.7 Conclusions

The above results show that the gene BCAL1092 of *B. cenocepacia* is an iron binding periplasmic binding protein that is processed from immature pre-peptide to mature *holo* iron binding protein when expressed in *E. coli*. Analytical size exclusion chromatography revealed that the protein product of BCAL 1092 is monomeric in solution and mass spectrometric analysis confirms the mass of the protein is that of the mature protein. As the coding DNA sequence in pET28a FbpA *B. cenocepacia* includes the wild type leader sequence characteristic of periplasmic binding proteins, this confirms that pre-protein is transcribed and that the N-terminal leader sequence is then cleaved to yield mature protein. Purified protein displays a LMCT absorption at 420 nm that is characteristic of iron binding proteins and ICP-OES analysis confirms that iron is indeed bound to the protein when purified from *E. coli*. This has led to designation of the protein product of BCAL1092 as FbpA *B. cenocepacia*

Native mass spectrometry studies of the *holo* protein suggest that FbpA *B. cenocepacia* preferentially utilises citrate as a synergistic anion when bound to Fe$^{3+}$. This observation was unexpected and pairwise amino acid sequence alignments with other known ferric citrate binding proteins from *E. coli* and *B. cereus* and *B. subtilis* reveal no significant amino acid sequence homology. To further confirm this finding, crystallisation of FbpA was attempted and has resulted in a 1.7 Å resolution structure that has a single iron atom bound to three tyrosinate oxygen atoms. As of yet, no exogenous anion ligand has been refined, but further work is being undertaken to solve this.

FbpA *B. cenocepacia* has no significant homology with other known ferric citrate binding proteins, but there are only a few examples of this type of protein currently available. Homologues of known ferric citrate transport systems are annotated as ferrichrome/ free Fe$^{3+}$ transport systems so bioinformatics analysis by function can be limited. FbpA *B. cenocepacia* was originally assumed to be a carbonate binding protein based on high sequence homology with FbpA *B. pertussis*. The x-ray crystal structure of this protein has already been solved (PDB2OWT) in complex with a carbonate anion. However this protein was expressed without its wild type periplasmic leader sequence, resulting in a cytoplasmic protein that was re-loaded with various anions post
purification (73). As this protein was never purified in *holo* form then it is feasible to suggest that carbonate is not the "native" synergistic anion in this case.

As the *Burkholderia* are natural plant pathogens, *B. cenocepacia* may have faced an evolutionary pressure to develop a ferric citrate ABC transport system in response to the use of ferric citrate as an iron storage system in plant xylem. In comparison the binding preferences of Fbp A *N. gonorrhoeae* and *H. influenzae* may reflect that these organisms are obligate human pathogens and as such have developed iron transport systems to suit a different environment. Furthermore, as iron acquisition is considered to be a virulence factor it could be suggested that alternative acquisition systems reflect alternate virulence pathways.

It seems that factors other than periplasmic environment are involved in anion utilisation in FbpA proteins. *Burkholderia* genus bacteria are plant pathogens and have evolved to colonise plants, unlike *N. gonorrhoeae* that is an obligate human pathogen. One significant difference between human and plant iron storage is the use of ferric citrate as an effective iron storage and transport system in the xylem. That *B. cenocepacia* expresses an iron transport system that is able to bind ferric citrate is perhaps a reflection of that ancestry.

Unfortunately, the original question of how iron is transported through the cytoplasmic membrane cannot be answered using the data in this body of work. The experimental midpoint potential of FbpA *B. cenocepacia* is not out with the range of physiological reductases but without correction for equilibrium coupled dissociation this cannot be stated with certainty.

### 4.8 Future Work

The next step in this research would be to assemble the entire *B. cenocepacia* J2315 ABC transporter *in vivo* and *in vitro*. *In vivo* assays for uptake of ferric citrate via isotopically labelled substrates would determine if the entire chelate complex is transported into the cytoplasm or if only iron is transported, leaving citrate in the periplasm. This experiment could directly address the model proposed for reductive release of iron from FbpA. If ferric citrate loaded FbpA interacts with the ABC transporter complex and releases citrate back into the periplasm or dissociates from the transporter complex as FbpA[Cit] then the proposed model for reductive release by
facile anion exchange is still valid. If the entire chelate complex is transported to the cytoplasm then it would appear that FbpA *B. cenocepacia* does not need to reduce ferric iron for transport to the cytoplasm (in the case of this transporter). Furthermore, whilst stable [FbpA+Fe+X] have been demonstrated and characterised electrochemically, it has not yet been proven that FbpA loaded with other synergistic anions is still able to transfer iron to the appropriate ABC transporter.

*In vitro* experiments with the ABC transporter reconstituted into proteoliposomes would achieve the above, but could also determine if the presence of a reductase is necessary for release of iron. If the reconstituted transporter performs *in vitro* transport then it would rule out the presence of an unknown reductase. Further experimentation would then have to be performed to ascertain if release of iron substrate is facilitated by conformational change induced upon binding to receptor TMD, or if a reduction mechanism is applied by the TMD itself. A *Mycobacterium tuberculosis* haem transporter (IrtA) contains a FAD binding domain.
Chapter 5  Large Scale Expression, Purification and Insolubility of Ferric Binding Protein C from N. *Gonorrhoeae*.

As FbpC has been previously characterised by this laboratory, and the x-ray crystal structure solved in collaboration with Dr. Simon Newstead (University of Oxford), expression plasmids were already constructed and expression protocols had been previously developed. The starting point for this project was to follow established expression methodology and purify fresh FbpC for kinetic studies with an aim to investigating a possible C-terminal regulated trans-inhibition mechanism.

### 5.1 Expression and Purification of Ferric Binding Protein C *N. gonorrhoeae*.

pET28a FbpC plasmid was transformed into BL21 (DE3) cells and overnight cultures were grown at 37°C in 250ml LB broth pH 7.5. These cultures were used to inoculate 5L TB (Terrific Broth) media, and protein expression was induced with 0.4mM IPTG at an O.D$_{600}$ of 0.8. The temperature was reduced to 25°C and the cells were left to grow for a further 18 hours overnight. Cells were collected by centrifugation at 5000 $g$ for 20 minutes at 4°C.

Cell pellet was resuspended in 25mM HEPES pH 7.5, 300mM NaCl, 10% Glycerol, 1mM ATP with DNase. Lysis was then performed by sonication on ice for 15 x 30 seconds, and cell free extract was prepared by centrifugation at 25,000 $g$ for 30 minutes at 4°C.

Supernatant was .45 μM filtered and injected onto a NiNTA HisTrap 1ml HP column (GEHealthcare) pre-equilibrated with re-suspension buffer (as above) with 20mM imidazole. Care was taken to perform all steps at 4°C. An elution gradient of 0-500mM imidazole, with a stepped phase at 60mM, was applied and fractions were collected and analysed by SDS PAGE (Figure 33).
Figure 33: (A) ÄktaTA FPLC Chromatograph of FbpC purification. Green line indicates the stepped elution gradient with a plateau at 60mM imidazole, before increasing to 500mM as a final step. The blue line is elution of proteins monitored by absorption at 280 nm. (B) SDS PAGE analysis of the AKTA FPLC purification of FbpC. FT is the flow through that did not bind to the NiNTA column, LMW is the protein marker, band sizes are written on the side of the gel. Wash is eluent from the column wash step. Numbers correspond to fractions collected in the chromatograph shown in A.

As illustrated in Figure 33 both peaks visible in the chromatograph contain a protein approximately 40 kDa in size. As FbpC has a molecular weight of 37744 Da this is in agreement with the purification of the protein in question. Fractions 11-22 were collected and concentrated by ultrafiltration to facilitate a second dimension of purification by size exclusion chromatography (SEC).

Unfortunately, during this concentration step the FbpC protein precipitated out of solution. FbpC is notably “sensitive” to high concentrations and imidazole, an observation made by previous colleagues that have worked with the protein. In this instance an adjustment to the purification protocol was determined that involved the removal of imidazole by dilution or dialysis immediately after IMAC purification. Therefore the purification had to be repeated, however the next time that the protein was purified it precipitated a step earlier, and crashed out of solution as it was purified off the IMAC column. This was rapid as precipitate forming was visible to the eye. To counter this, it was decided that whilst imidazole (or perhaps histidine) must be used to compete the protein from the Ni.NTA matrix, that the effects of high concentration could be mediated by diluting the elution fractions immediately into non-imidazole containing buffer. Sadly this could not be implemented because soluble expression of the protein could no longer be achieved.
5.1.1 The Insolubility of Ferric Binding Protein C.

After the protein precipitated during the purification process it was necessary to culture more *E. coli* cells expressing the protein. Despite replicating the expression conditions outlined previously FbpC was repeatedly expressed in the insoluble fraction usually in aggregate inclusion bodies. This was typified by a white pellet, characteristic of this type of aggregated protein expression and SDS PAGE analysis after cell lysis revealed a highly expressed protein at 37 kDa that is pelleted with the cellular debris (Figure 34).

![SDS PAGE Analysis](image)

**Figure 34: SDS PAGE Analysis of lysed *E. coli* BL21(DE3) cells expressing pET28a FbpC.** Pellet represents the cellular debris pelleted after lysis, as well as the insoluble fractions of the cell. S/Nat is the soluble component of the cell free lysate and is fraction that contains soluble protein. FbpC is visible as an overexpressed protein in the cell debris pellet. LMW is the protein marker used.

We were unable to extract any active FbpC from the soluble fraction of these cells. The expression was repeated, under identical conditions (several times), to ensure that no unconscious error had been made but this was futile as the protein was never soluble (in my hands) under these conditions again.

5.1.2 Optimisation of FbpC *N. gonorrhoeae* expression conditions.

As expressed FbpC was now insoluble for an undetermined reason the first step that was taken toward addressing this problem was varying expression conditions.

When optimising the expression of a protein, there are many different aspects of the protocol that can be varied. Some of these are; varying the host strain used as an expression vehicle, the media that the strain is cultured in, the temperature that the
cells are cultured at and also the concentration of the chemical used to induce protein expression (if behind and inducible promoter, as is the case with FbpC). Below a table summarises the expression conditions that were applied to FbpC.

Table 2: Expression conditions tested for expression of the construct pET28a FbpC in E. coli.

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Media</th>
<th>Temp °C</th>
<th>Induction (Hrs.)</th>
<th>IPTG (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21 [DE3]</td>
<td>TB (Home)</td>
<td>25, 30, 37</td>
<td>18, 6, 3</td>
<td>0.4, 0.1, 0.05</td>
</tr>
<tr>
<td></td>
<td>TB (EZ Express)</td>
<td>25, 30, 37</td>
<td>18, 6, 3</td>
<td>0.4, 0.1, 0.05</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>25</td>
<td>18,3</td>
<td>0.4, 0.1, 0.05</td>
</tr>
<tr>
<td></td>
<td>2YT</td>
<td>25</td>
<td>18,3</td>
<td>0.4, 0.1, 0.05</td>
</tr>
<tr>
<td></td>
<td>Overnight Express™ Instant</td>
<td>25, 30, 37</td>
<td>18,3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMS174</td>
<td>TB (Home)</td>
<td>25, 30, 37</td>
<td>18,3</td>
<td>0.4, 0.1</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>25</td>
<td>18</td>
<td>0.4</td>
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Variations in the temperature used in expression conditions can alter the expression profile of a protein by slowing down the metabolism of the host cell. Varying IPTG concentration (in this case it was reduced from the original protocol) affects the rate at
which the cell is forced to express protein. Cellular homeostasis is tightly regulated and using IPTG to induced protein expression forces the cell to divert critical resources toward the expression of the protein. This is not good for the host and in some cases it cannot tolerate a high level of expression resulting in protein aggregates, inclusion body formation and possibly cell death. By reducing IPTG concentration and incubation temperature then protein expression may proceed at a slow pace, but with successful expression (97).

Another avenue to explore is the host strain used to express the protein. E. coli BL21 [DE3] are the standard strain used in this laboratory, but several other mutated derivatives are available. BL21 DE3] Codon Plus harbour a plasmid that encodes for low usage codon and can avoid translational issues if a sequence is not codon-optimised toward the host cell(98). If the sequence being expressed codes for low usage tRNAs then transformation into a strain such as Codon Plus may enhance expression by providing rare tRNAs.

Though the literature protocol states that BL21 [DE3] E. coli were used to express FbpC it is known that membrane-associated proteins can be toxic to the strain. A BL21 derivative, C43 [DE3] cells were transformed with pET28a FbpC and used to express the protein. C43 cells carry a double mutation that manifests in a greater expression capability toward ATPase's (99). Despite this, the protein was still insoluble after expression.

Unfortunately, none of the above conditions resulted in soluble protein expression. At this juncture, there are many paths that deal with insoluble protein. Even though the protein is known to be “temperamental” to those that work with it, FbpC has still been successfully purified and had the x-ray crystal structure solved. Somewhat frustratingly, this has been done by previous members of this laboratory and even the passed down, “bench wisdom,” that people develop as they get to known their target, was not enough to get FbpC soluble again. Given that the protein had been soluble in my hands we therefore chose to eschew more complex options, such as re-cloning or purchasing a synthesised, codon optimised gene.

Next, a systematic variation in the mechanical handling of the whole cell pellets during the lysis procedure was undertaken. Lysis by sonication, cell disruption, freeze/thaw cycles was attempted with no success. Enzymatic lysis with lysozyme and also chemical
lysis using commercially available reagents such as BugBuster were also futile. At no point was FbpC present in the soluble fraction.

Following this, changing the composition of the lysis buffer was trailed. ATPase enzymes often require ATP in buffers to assist in stabilising the protein of interest. ATP concentration was varied from 0-10mM but did not make any difference. Ionic strength (NaCl) was varied 0-0.5M, glycerol and the detergents Triton x-100 and n-Dodecyl β-D-Maltopyranoside (DDM) were added to lysis buffers, but no soluble FbpC was ever extracted. At this point, it was decided to attempt purification of FbpC from fermented cultures that had been used to provide the protein used in x-ray crystallography experiments. It was hoped that has this protein had been previously purified successfully, that the current problems with insolubility could be circumnavigated.

5.1.3 Purification of Ferric Binding Protein from a Fermentation Culture.

Simon Newstead, whom had previously used pellet from this culture to extract the protein, gifted fermented cell pellets expressing the FbpC protein. The culture was prepared in 2009 (2.5 years previous to this work) and had since been stored at -80 °C.

Fermented pellet was re-suspended in 25mM HEPES (pH 7.5) with 0.3mM NaCl, 10% Glycerol and 1mM ATP before lysis via cell disruption (25 k PSI). The cell debris was pelleted and the supernatant applied to NiNTA resin that had been equilibrated with resuspension buffer plus 20mM imidazole. A batch gradient of 60mM, 100mM and 250mM imidazole was used to elute proteins into re-suspension buffer without imidazole (diluting imidazole concentration to prevent precipitation). This dilution step was factored in as FbpC can be sensitive to high imidazole (> 250mM) concentrations. Samples were collected and analysed by SDS PAGE.

As can be seen in Figure 35 most of the protein was in the cell debris pellet that was collected by centrifugation, although a small amount of protein also appeared in the 100mM and 250mM soluble imidazole fractions. These fractions were dialysed into 10mM HEPES (pH 7.5), 1mM ATP, 10% Glycerol and 0.5mM EDTA. The addition of the chelator was to inhibit ATP hydrolysis as FbpC requires Mg$_2^+$ as a cofactor. In preventing the enzyme from turning over it may help increase stability thanks to ordered structure around the substrate binding site.
**Figure 35: SDS PAGE Analysis of FbpC purification.** Whole Cell is a sample of cells prior to any lysis. Snat is the supernatant. LMW is low molecular weight marker and FT is the flow through after binding to resin.

Each of the dialysed fractions were re-examined by SDS PAGE to assess purity. Protein concentration was approximately determined by measuring the relative absorbance at 280 nm by UV Visible Spectroscopy.

**Figure 36: SDS PAGE Analysis of FbpC purification fractions.** LMW is the protein marker used in the gel. Wash and 100mM refer to the fractions visible in Figure 35 that have been concentrated by ultrafiltration through a 30 kDa molecular weight cut off membrane.

Despite low homogeneity, the purified protein fractions collected were assayed for ATPase activity. Two assays were used but no activity was detected, and is likely due to the age of the fermented cell pellets.

5.1.4 Re-cloning of the FbpC Expression Vector.

Faced with insoluble protein it was decided that re-cloning the expression construct into another vector might help overcome the problem. pETHISTEV, a pET30 derived plasmid, was kindly donated by the Naismith group (University of St. Andrews). The plasmid encodes an N-terminal His₆ tag with a TEV protease recognition sequence
located between the tag and the multiple cloning site (MCS). It was designed to offer a removable N-terminal His tag that could be removed post purification for optimized crystallographic conditions, but had also been noted that some proteins expressed with a higher efficiency in this plasmid. Therefore FbpC was re-cloned into pETHISTEV in the hope that this effect may also occur in this case.

FbpC was excised from pET28a by restriction digestion using NcoI and XhoI, purified by 1% agarose gel electrophoresis and directly ligated into pETHISTEV vector digested with the same restriction enzymes. Successful construction of the new expression vector was confirmed by Sanger di-deoxy sequencing and restriction digestion analysis.

pETHISTEV FbpC was transformed into BL21 [DE3] competent cells and selected for on kanamycin LB Agar. A single transformed colony was used to inoculate a 250ml LB culture and shaken at 37°C overnight. This was back diluted into 50ml TB media and protein expression was induced by the addition of 0.05-0.5mM IPTG. Expression was monitored at 3 hours, 6 hours and 18 hours. Expression of the protein was detectable by SDS PAGE analysis.

Small-scale lysis was performed by the addition of lysozyme to cell pellets, on bench incubation for 30 minutes, followed by sonication for 15 minutes. Cell free extract was collected by centrifugation and analysed by SDS PAGE. Over expressed protein was visible only in the pellet, meaning that none of the protein was in the soluble fraction of the cell.

5.1.5 One last attempt: Expression and Purification of Ferric Binding Protein.

After numerous attempts at expressing FbpC as a soluble protein, a final attempt was made in collaboration with Dr Simon Newstead. This expression and purification was carried out in Simon’s lab in the Biochemistry Department at Oxford University as a final attempt made under the guidance of someone who had previously purified the protein successfully.

pET28a FbpC was used to transform C43 and BL21 [DE3] competent cells and a 3L TB culture was grown. The cell pellets were re-suspended as outlined before and lysed by cell disruption at 40 kPSI before centrifugation at 20000 g and a second ultracentrifugation clarification step at 141,000 g. A HisTrap 5ml NiNTA column was
used to purify the protein, but after this step it was clear that there was no protein bound to the column. SDS PAGE analysis revealed that, once again, the protein had been expressed but was contained within the cellular debris after centrifugation.

At this point, the next logical step would be to create an N-terminal fusion with a high solubility partner. There are various options that would be suitable and are commercially available, such as maltose binding protein (MBP) or *E. coli* thioredoxin. Both MBP and thioredoxin are soluble and are also translated at high efficiency. By fusing the N-terminal of FbpC to one of these proteins then you are trying to create a system by which the cell will tolerate a high concentration of your target protein. A proteolysis site can be engineered to allow cleavage of the fusion partner once the target has been purified.

5.1.6 111 Amino Acid C-terminal Truncation of Ferric Binding Protein C.

The crystal structure of FbpC revealed that each monomer has a 240 amino acid domain with typical ATPase structural motifs(43). There is also a 110 amino acid C-terminal domain, which has been observed in other NBD proteins (100-101) but is not uniform throughout ATPase enzymes. The C-terminal domain of FbpC is unusual in that when dimerised it undergoes a domain swap which has not been previously observed in an ABC transporter. Within this domain is a cavity that contains a cluster of six histidine residues.

We wished to investigate the role of the observed histidine motif as well as the C-terminal domain in entirety. To this end a 111 amino acid C-terminal truncation of FbpC was designed which would cause deletion of the entire regulatory domain.

The truncated FbpC was created by PCR using primers that had been designed to complement the DNA sequences encoding the N-terminal and 238-241 amino acids of FbpC. An *NcoI* restriction site was engineered into the 5’ primer to complement the start codon and facilitate cloning into pET28a plasmid. At the 3’ a stop codon and *XhoI* restriction site created the truncation fragment and restriction site for ligation into pET28a. This plasmid was chosen on the suitability of restriction sites that were available.
Figure 37: Plasmid map of pET28a FbpC Δ 111 Truncation. The plasmid encodes for the first 241 amino acids of FbpC from *N. gonorrhoeae*. A kanamycin marker allows for selection and protein expression can be induced by IPTG induction under control of the T7 promoter.

PCR was performed using Platinum® Pfx DNA Polymerase (Invitrogen) and product was examined by 1% agarose gel electrophoresis. An amplified band at ~ 730 base pairs corresponded to the expected fragment size of 723 base pairs and so this was excised and gel purified via the QIAquick Gel Extraction Kit (Qiagen). Taq polymerase (PuReTaq Ready-To-Go PCR Beads GE Healthcare) was used to poly-adenylate the blunt ended PCR fragment in preparation for ligation into pGEM®-T Easy Vector (Promega). Successful ligations were selected for by blue/white screening on S-gal agarose (GEHealthcare), picked into overnight LB broth cultures before pGEM plasmid DNA was extracted by mini-prep (QIAprep Spin Miniprep Kit – Qiagen). *Ncol/Xhol* digestion of pGEM resulted in excision of the FbpC truncation fragment from the vector and this was again gel purified prior to ligation (T4 DNA ligase NEB) into *Ncol/Xhol* linear pET28a backbone vector. Restriction digestion analysis and Sanger dideoxy sequencing confirmed to construction of the truncated FbpC pET28a expression vector. The constructed was named pET28a FbpC Truncation.

pET28a FbpC Truncation was used to transform *E. coli* BL21(DE3) competent cells and transformants were selected for on LB Agar containing kanamycin antibiotic. 20ml LB broth liquid cultures were inoculated with a single colony and incubated at 37°C overnight. The following morning, 4 x 100ml LB cultures were back diluted to O.D600 =0.1. Protein expression was induced at O.D600 =0.6 using 0.1, 0.5, 1.0 and 2.5 mM IPTG and allowed to incubate at 30°C for 3 hours.

Whole cell pellets were resuspended in lysis buffer composed of 25mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5mM ATP, 20% glycerol and a Roche Protease Inhibitor Tablet.
Lysis was performed by sonication on ice (30x30 for 10mins) and the cytoplasmic fraction was isolated by centrifugation. SDS PAGE analysis revealed that ΔFbpC had expressed but was entirely present in the insoluble cell debris after centrifugation.

At this point it was decided that as all FbpC N. gonorrhoeae proteins had expressed as insoluble for a long period of time, then it would be wise to cease work on this area. As of the time of writing, FbpC is still insoluble and the problem has not been solved. If work were to be continued in this area then designing a synthetic gene codon-optimised for expression in E. coli would be the next step.

5.1.7 Cloning and Expression of BCAL1090 Putative FbpC B. cenocepacia.

Identification of a 3 gene ORF in the B. cenocepacia J2315 genome was consistent with a putative iron binding ABC transporter. The three genes, BCAL1092, BCAL 1091 and BCAL1090 code for the PBP, TMD and NBD domains respectively and has BCAL1092 has been confirmed as an iron binding PBP, attempts were made to express and purify BCAL1090 to confirm ATPase NDB activity. Furthermore, amino acid sequence alignments between BCAL1090 and FbpC N. gonorrhoeae reveal that BCAL1090 is 109 amino acids shorter (Figure 18) than FbpC N. gonorrhoeae and lacks the theoretical C-terminal regulatory domain that has been identified in the latter.

A synthetic gene, codon-optimised for expression in E. coli was purchased from GenScript (USA) and sub-cloned via 5’NcoI and 3’XhoI restriction sites in the pET30 derivative pET HIS-TEV. This plasmid encodes for a N-terminal His<sub>6</sub> tag followed by a TEV protease epitope that allows enzymatic cleave of the His tag and liberates the protein of interest after purification is complete. The coding DNA sequence is under control of the T7 promoter and so protein expression can be induced with IPTG in any compatible [DE3] E. coli cell. Correct construction of pETHISTEV BCAL 1090 was confirmed by restriction digestion analysis.
Figure 38: Plasmid map of pETHISTEV BCAL 1090 (FbpC NBD from Burkholderia cenocepacia). A kanamycin marker allows for selection and protein expression can be induced by IPTG induction under control of the T7 promoter. A N-terminal His6 tag followed by a downstream TEV protease epitope allows removal of His tag post purification.

pETHISTEV FbpC B. cenocepacia was used to transform E. coli BL21[DE3] competent cells and transformants were selected for on LB Agar containing kanamycin antibiotic. 20ml LB broth cultures were inoculated with a single colony and incubated at 37°C overnight. The following morning, 4 x 100ml LB cultures were back diluted to $O.D_{600} = 0.1$. Protein expression was induced at $O.D_{600} = 0.6$ using 0.1, 0.5, 1.0 and 2.5 mM IPTG and allowed to incubate at 30°C for 3 hours.

Whole cell pellets were resuspended in lysis buffer composed of 25 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM ATP, 20% glycerol and a Roche Protease Inhibitor Tablet (EDTA free). Lysis was performed by sonication on ice (30x30 for 10mins) and the cytoplasmic fraction was isolated by centrifugation. SDS PAGE analysis revealed that FbpC B. cenocepacia had expressed but was entirely present in the insoluble cell debris after centrifugation.

5.2 Discussion.

The aim of this section of work was to purify FbpC N. gonorrhoeae and a truncated mutant with regard to investigating the possibility that the C-terminal 110 amino acid domain of the protein has a regulatory role. Unfortunately this was not possible as soluble, active protein was not obtainable. Somewhat frustratingly, FbpC N. gonorrhoeae has been purified and the x-ray crystal structure solved and this work was done using the same expression construct that was used in this body of work. Initially, FbpC was soluble, but assays were not performed using this protein as it precipitated
out of solution during initial purification stages. After this, soluble expression of the protein was never again achieved, despite a huge variation in expression conditions and considerable period of time dedicated to solving the problem. Even going so far as travelling to the laboratory of crystallographer whom previously purified and solved the structure of the protein did not yield soluble protein. If FbpC has not previously expressed in the soluble fraction, then re-cloning and codon-optimisation would have been logical first steps to addressing insolubility but because soluble protein had been obtained before it was felt that this was not the problem and so time was sent working on optimising expression conditions. Sadly, this was fruitless and it was felt that a line should be drawn under attempts to work with this protein.

Other FbpC constructs, including a N. gonorrhoeae 111 amino acid C-terminal truncation and a putative FbpC homolog from B. cenocepacia were also cloned and subjected to expression testing. Original expression screens showed that both of these proteins were also insoluble, and given the problems experienced with FbpC N. gonorrhoeae it was decided that optimisation work on these proteins was not to be attempted. Instead, other projects were pursued.

If the work were to be repeated, with the knowledge gained from the above experience, I would immediately move to re-cloning the constructs and not spend time optimising expression conditions. Other possibilities include purifying inclusion bodies and attempting re-folding of the protein via serial dilution but protein yields are significantly lower using this method and the methodology is far more complex. As FbpC had been previously soluble, re-folding was avoided in this body of work, as it was felt that the problems experienced were caused by a problem during the expression stage.

It remains to be seen if FbpC N. gonorrhoeae and other derivatives can now be expressed as outlined in the literature.
Chapter 6   Expression and Purification of Thioredoxin-HBD2

Several recent publications have implemented the use of commercially available thioredoxin fusion systems as a vector for expression of soluble and bioactive beta defensin peptides. Antimicrobial peptides purified in this manner are human beta defensins 26 & 27, Plactasin as well as HBD2 in which all groups have reported successful expression of a soluble fusion protein which does not possess antimicrobial activity \((143,144,145)\). The ability of the cleaved peptides to kill bacteria was also established, as well as chemotactic activity for those that possess it, leading to the thioredoxin fusion peptides earning the label, “bioactive.” There has, as of yet, been no structural characterisation performed on any of the reported peptides purified in this fashion and therefore it is not understood how thioredoxin (Trx) fusions are able to counter the antimicrobial properties of defensins. Plasmids that encode Trx fusion tags have been available commercially for some time as a method by which to express toxic and insoluble proteins.

Trx is ubiquitous through a variety of organisms where it acts to reduce cytosolic proteins via cysteine thiol-disulfide exchange and plays an important role in cellular redox regulation. \(E.\ coli\) possess a native Trx that is natively expressed as a cytoplasmic protein and it is this version of the protein that has been exploited as a fusion tag in the aforementioned commercial vectors. Advantages inferred by this are that \(E.\ coli\) is able to tolerate a high level of Trx (up 40% of cytosolic protein content) resulting in a higher yield of fusion protein per gram of \(E.\ coli\) biomass.

A soluble fusion able to produce milligram quantities of protein was an attractive proposition, and so a thioredoxin HBD2 fusion protein was designed. A thioredoxin fusion vector, pET32b, was purchased from Novagen® which encoded an N-terminal thioredoxin with a central hexahistadine (His\(_6\)) tag followed by an enterokinase cleavage site and MCS (multiple cloning site) We wished to incorporate a TEV cleavage site at the N-terminal of HBD2 as we are able to produce TEV protease using recombinant methods and this provided us with a cheap alternative to enterokinase.
6.1.1 Cloning and Expression of Thioredoxin-HBD2 Fusion Protein.

Figure 39: Plasmid map of pET32b His-Trx-TEV-HBD2 and the amino acid sequence of the encoded fusion protein. (A) pET32b Trx-TEV-HBD Vector NTI plasmid map. Restriction sites used to clone in both the TEV-HBD2 and Trx-TEV-HBD2 synthetic genes are indicated. pET32b encodes for resistance to ampicillin and protein expression can be induced by addition of IPTG via lacL. (B) Amino acid sequence of the entire Trx-TEV-HBD2 construct and (C) a schematic drawing diagram of the designed construct. Red represents His$_6$ tags, Cyan is Trx, Silver is the TEV protease epitope, green is the redundant enterokinase epitope and finally HBD2 is represented by dark blue.

A 126bp synthetic gene that encoding for HBD2 when a TEV protease epitope at the immediate N-terminus was purchased from Clontech Inc. TEV protease recognises the amino acid sequence ENLYFQG and results in cleavage between the latter glutamine and glycine residues. As the first amino acid of mature HBD2 is glycine this construct will result in HBD2 without any N-terminal extension, something that was highly desirable given the small size of the peptide.

The TEV-HBD2 gene was designed with the restriction sites BamHI and HindIII at 5’ and 3’ ends respectively. pET32b and pUC57 TEV.HBD2 were digested, gel extracted
and ligated before transformation into high competency *E. coli.* Despite several attempts at this ligation, with varying ratios of vector to insert, we were unable to ligate the gene into the vector. This difficulty has been attributed to the 126bp synthetic gene and the 29bp excision fragment of the vector plasmid being almost impossible to visualise by agarose gel electrophoresis.

To increase both the size of the gene and also the size of the excision fragment in the vector plasmid, the gene was redesigned to incorporate the entire Trx tag. pET32b possesses a *XbaI* site ~ 40 bps upstream of the tag and so a new synthetic gene (Clontech), encoding for the whole Trx.TEV.HBD2 fusion construct was designed to insert between 5’ *XbaI* and 3’ *HindIII* restriction sites.

Digestion, extraction and ligation were performed as mentioned previously, and transformed colonies were sequenced to confirm insertion of the gene. This new fusion protein was then expressed in BL21 [DE3] cells and protein expression was induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

**Figure 40:** SDS PAGE Analysis of Trx-HBD2 fusion protein in *E. coli* BL21 [DE3]. **A:** Whole cell analysis of *E. coli* BL21 [DE3] transformed with pET32 Trx-HBD2 fusion, under 0.5mM IPTG protein expression induction after 3 hours at 37°C. **B:** Whole cell analysis of BL21[DE3] cells transformed with pET32b Trx-HBD2 in the absence of IPTG.

SDS PAGE analysis of intact cells was performed to ascertain if the Trx-HBD2 fusion had been successfully expressed in the bacterial cells. Figure 44 (A) shows the presence of an overexpressed protein band at approximately ~26 kDa. The expected molecular mass of the Trx-HBD2 fusion protein was expected at ~22 kDa but it is not unusual for
proteins to run slightly anomalously on this type of gel. As a clear over expressed band was visible, purification of the protein was attempted.

6.1.2 Purification of Thioredoxin-HBD2 Fusion Protein.

10g of cell pellet was harvested by centrifugation, before re-suspension in 50mM Tris-HCL pH 7.5 with a complete™ EDTA-free Protease Inhibitor Cocktail Tablet (Roche) added. It should be noted that protease inhibitor tablets are effective against serine proteases and as TEV is a cysteine protease there is no risk of inhibition. Lysis was performed by sonication, and not by the addition of lysozyme, which is commonly used to disrupt *E. coli* cell membranes. Lysozyme is small (14 kDa) and has a similar cationic charge to HBD2 and it was felt it would unnecessarily complicate the purification protocol if lysozyme were added to the protocol.

Our original experimental design for purification was based upon several published articles that utilised affinity chromatography. Initially, the first dimension of purification was Immobilised Metal Affinity Chromatography (IMAC) using either Fastflow Ni-NTA beads (Qiagen) or Ni-NTA HP Äkta columns (GE Healthcare) but this resulted in a substantial loss of protein (Figure 41) that would not bind to the resin. Cell free extract was allowed to incubate on 1-10ml Ni-NTA resin before elution of bound protein by an increasing step-wise gradient of imidazole. (Figure 44). TALON® Metal Affinity Resin which is a cobalt affinity resin was also tested in the same fashion. Whilst there is purified protein visible (predicted weight ~23 kDa) in Figure 44(A) this is a concentrated sample and the majority of protein remains in the unbound flow through. Cobalt resin, which has a higher specificity for His<sub>6</sub> tags but lower binding capacity, also had the same effect (Figure 44(B).
Figure 41: SDS PAGE analysis of Ni-NTA Resin (A) and Cobalt (B) resin purification of Trx.TEV.HBD2 fusion protein. LMW = Protein marker. FT = flow through and imidazole concentration (mM) used in batch elution is written above each sample.

This was attributed to a high concentration of fusion protein in the cell free extract, combined with a high level of cellular proteins and so ion exchange (IEX) chromatography was applied in place of IMAC as an enriching step when working with Trx.TEV.HBD2. The isoelectric point (pI) was calculated using Vector NTI Advance 11.0 and was determined to be 6.20. Anion exchange chromatography using a 26/10 Q-Sepharose (NH₄⁺) High Performance (GEHealthcare) column acted to enrich for the over expressed 22870 Da fusion protein. (Figure 42)

60ml of .22 uM filtered cell lysate was loaded applied to the column that had been pre-equilibrated with 50mM Tris-HCl buffer pH 8.0. A 0-1M NaCl isocratic elution gradient was applied. Protein elution was monitored at 280 nm.

Fractions containing sample (1-26) were analysed for fusion protein by 12% SDS PAGE analysis (Figure 46). A second peak visible in the chromatograph between fractions 27-40 was expected, and is known to be DNA.
Trx.TEV.HBD2 has a predicted Mr of 22870 Daltons and over expressed fusion protein is visible in pooled fractions 7-18. To further determine which fractions contained the protein of interest these fractions were then analysed individually via SDS PAGE.

Fractions 9 – 16 appeared to contain high quantities of fusion protein, albeit with high levels of background cellular proteins which was something that was anticipated due to a pI similar to many other *E. coli* proteins. These fractions were concentrated through a 10,000 Da molecular weight cut off (MWCO) spin filter and then 20µg of fusion protein, concentration was calculated by 280 nm absorbance, presuming that 1 absorbance unit (A.U.) is equal to 1mg/mL of protein, was cleaved with TEV protease. Samples from the cleavage reaction were taken over a time course and these were analysed by SDS PAGE.
6.1.3 TEV cleavage of Thioredoxin-HBD2 fusion protein.

TEV cleavage results in a ~ 5 kDa loss in the size of the fusion protein when analysed by SDS PAGE which corresponded with the loss of the 4330 Da HBD2 peptide. The active site of TEV protease includes a catalytic cysteine that participates in hydrolysis of the peptide backbone, becoming oxidised in the process. Cleavage conditions require addition of a reducing agent, such as dithiothreitol (DTT), which acts to reduce the catalytic cysteine residue and restores function to the enzyme. As defensins are typically oxidised, ideally chemical reduction should be avoided and so to avoid the addition of strong reducing agents the cleavage reaction was performed without the addition of DTT. As TEV protease is purified with DTT in the buffers, we increased the ratio of protease to fusion protein in the hope of adding enough protein as to negate the need for a reducing agent (as without a reducing agent, each TEV protease would oxidise after a single catalytic turnover). A reaction containing DTT was also set up as a control, and also a Serine Palmytol Transferase (SPT) fusion protein that contains a TEV cleavage site, which is known to cleave successfully, was ran as a positive control (Figure 44).

TEV cleavage was allowed to proceed at room temperature for 30 hours. There is no reported substrate inhibition of TEV protease and so excess of the protease was not a concern. Therefore v/v ratios of TEV/ Substrate (Trx.TEV.HBD2 IEX purified fractions) were used to optimise cleavage conditions. As a negative control a sample was left in cleavage buffer without the addition of TEV protease for the reaction duration.
Figure 44: SDS Page analysis of TEV Cleavage of Trx.TEV.HBD2. LMW = Low Molecular Weight Marker. SPT control positive control reaction contained a 98 kDa fusion protein with TEV protease epitope. This fusion protein has successfully cleaved by TEV into two 49 kDa monomers. TEV protease is visible in the SPT control at ~ 30 kDa. A further positive control for the inclusion of the reducing agent DTT was included. Reactions, excluding DTT, were set up with decreasing Trx-HBD2:TEV ratios and a large scale (5ml) reaction ran alongside for preparatory purposes.

Figure 44 illustrates the SDS PAGE analysis of the cleavage reaction. The SPT control has been cleaved from a 92 kDa fusion to two monomers visible on the gel at ~ 46 kDa. The TEV protease is also clearly seen at ~ 30 kDa which corresponds to the size of the protein. The DTT buffer control shows three distinct bands at ~ 28 kDa, ~19 kDa and ~ 5 kDa. The first of these is TEV protease and the latter two are the Trx fusion partner and HBD2 peptide respectively. Compared with the “No TEV” negative control, of which there is one large band that is un-cleaved fusion protein, it is clear to see that both the DTT and the non-TEV cleavage reactions have worked, albeit the latter with less significantly less efficiency.

6.1.4 HBD2 purification by Cation IEX Chromatography

The next step at this point was to purify the cleaved peptide from the rest of the reaction mixture. Due to the cationic charge of HBD2 (pI 9.30 at pH 7.5/+6) IEX chromatography was the first method attempted. The crude mixture was loaded on to a HiPrep FF SP Strong Cation Exchange (SO3-) column pre-equilibrated with 50mM Tris pH 8.0. A 0-1M NaCl gradient was used to elute proteins and all fractions assayed for HBD2. Due to a lack of tryptophan residues, HBD2 cannot be reliably monitored at 280 nm and as such all fractions must be examined by SDS PAGE. Furthermore, the
recombinant form of TEV protease used in this experiment possesses a poly-Arginine tag that was designed for purification by cation exchange chromatography, meaning that there was a possibility that the two proteins would purify together.

SDS PAGE analysis confirms that TEV protease is present in fractions 7-20 (Figure 45). HBD2 is also seen in fractions 1-9 (Figure 47) but as a weak band visible on the gel. The peptide appears to have been eluted almost immediately off the column, which was not expected given its isoelectric point. As the point of this project was to develop a rapid and efficient method for purification of the peptide another method of purifying the cleaved peptide was required.

Figure 45: SDS PAGE Analysis of cation exchange (IEX) chromatography Trx-HBD2 TEV cleavage reaction. (A) AKTA FPLC chromatograph of cation IEX run. (B) SDS PAGE analysis of the fractions eluted from the IEX run. S/nat refers to the crude cleavage mixture prior to loading on to the IEX column. F/T is the eluent that did not bind to the column. Numbers refer to the fractions that correspond with chromatograph shown. TEV protease is visible as a ~25 kDa band in fractions 7-20.

6.2 Redesign of Trx-HBD2 fusion protein to incorporate an N-terminal His\textsubscript{6} tag.

The previously designed thioredoxin-HBD2 fusion protein contained a central his\textsubscript{6} tag that proved to bind to Ni-NTA resin with low affinity, resulting in a relatively crude protein mix after affinity chromatography. This suggested that this His\textsubscript{6} was not sufficiently exposed to allow binding to the affinity resin. Despite efficient cleavage of the fusion, purification of HBD2 was not achieved through cation IEX, despite a pI of 9.30. Given the small size and lack of absorption at 280 nm (the peptide does not possess the aromatic residue tryptophan) it was decided that improving the first
dimension of chromatography would result in a cleaner extract from which to purify cleaved defensin, making the second dimension easier.

To satisfy this goal, a further gene was designed and purchased (Clontech), this time incorporating an N-terminal His6 tag, as well as the central one.

![Diagram](image)

**Figure 46**: Schematic of the N-terminal his tagged thioredoxin-HBD2 fusion protein.

The 732bp construct was excised from pUC57 using a double *XbaI* and *HindIII* restriction digest, before ligation into a pET32b vector previously restricted with the same enzymes. Sanger sequencing confirmed insertion of the cassette, and the newly constructed HTTHBD2 plasmid was transformed in *E. coli* BL21 (DE3) competent cells for protein expression.

#### 6.2.1 Purification of His-Thioredoxin-TEV-HBD2 fusion.

The new fusion protein was expressed as previously detailed. IMAC chromatography was performed as before: On an AKTA FPLC using a GE Healthcare 1ml Ni-NTA HiTrap column. The cell-free extract was loaded onto the column, and an imidazole gradient of 20 – 500mM with an extended 60mM washing step was applied. Fractions were monitored at 280 nm and further analysed by SDS PAGE. This showed that, despite two histidine tags fusion protein had eluted across the entire gradient with considerable contamination. Despite this contamination, TEV cleavage was performed on the fractions containing fusion protein.
Attempts to purify HBD2 were made using a heparin based affinity chromatography method. Heparin, a glycosaminoglycan, has an anionic charge and has also been shown to complex with HBD2 (146). In this instance heparin could act as either an affinity/ IEX chromatography step.

A 1ml HiTrap Heparin HP (GEHealthcare) column loaded with cell free lysate was subjected to elution conditions of 0.1 – 2M NaCl. Fractions analysed by SDS PAGE. Both HBD2 and the fusion partner were eluted between 100-500mM NaCl, which was unexpectedly low for HBD2. There was no separation of HBD2 from TEV protease, and it was clear that a significant amount of peptide was lost in the process. The use of heparin affinity/ IEX chromatography as a means of extracting HBD2 was discarded as inefficient.

The difficulty in purifying HBD2 peptide from a relatively crude mixture had been a stumbling block on several occasions by this point and so it was decided that further optimisation of the first chromatography step might be a better way to approach the problem. To this end different Ni-NTA affinity resins were tested using batch elution methodology (instead of FPLC).

Nickel and cobalt resins (cobalt has less binding capacity but greater specificity) from Invitrogen, Qiagen, Promega and Clontech were tested. Invitrogen and Qiagen resins were better able to purify the fusion protein, resulting in a cleaner first chromatography step. The elution gradient was also refined to give a final washing step...
of 100mM imidazole before elution with 500mM resulting a single step elution. Unfortunately, as shown in Figure 47 there was still contamination after purification, but significantly less than with previous methodologies.

A

B

Figure 48: (A) Batch IMAC purification of HTTHBD2 fusion protein. LMW is the protein marker, F/T is the flow through from the resin and the concentration of imidazole used to elute in a stepwise fashion is written given in mM. (B) TEV Protease cleavage of HTTHBD2 fusion protein.

Still, the problem of purifying the peptide from the cleavage mixture persisted. As trying to purify the peptide had failed to pull it cleanly out of solution, we decided instead, to try to not purify the peptide, but to get rid of everything else instead. Following TEV cleavage, the samples were re-incubated with Ni-NTA (Qiagen) resin in a second IMAC step. The flow through was collected, and the resin was washed with a 20-500mM imidazole gradient. Fractions were again analysed by SDS PAGE, and this time the unbound flow through contained HBD2 peptide (Figure 49).

Figure 49: SDS PAGE of Purified HBD2. Before and after TEV cleavage samples show the intact fusion protein before it is cut and then then cleaved sample that the peptide was purified from. FT is the unbound flow through and lanes 1-4 are increasing imidazole concentrations. 1=20mM, 20=50mM, 3= 100mM and 4 = 500mM.
TEV protease contains a His<sub>6</sub> tag so would be retained on the resin. Also, the other contaminants from the first chromatography step would be retained on the resin as well. In fact everything bar HBD2 bound to the resin, whilst the peptide was contained in the flow though off the column. This method of reverse purification negates the problem of TEV protease binding to cation exchange columns as well as removing contaminants that bound to the first Ni-NTA column and results in a quick and easy method for the purification of HBD2.

### 6.2.2 LC-ESI-MS Analysis of HBD2 containing fractions.

Fractions that were judged to contain HBD2 by SDS PAGE analysis were submitted for LC-ESI-MS analysis (Dr. David Clarke, SIRCAMS, Edinburgh). From the first successful HBD2 purification, protein concentration was determined as 37.5µg/mL or 8.5µM by bicinchoninic acid (BCA) assay. Measuring absorbance at 280 nm is reported to be inaccurate due to the low number (3) of aromatic residues in HBD2. The presence of cysteine residues is known the increase the sensitivity of the BCA assay.

![Figure 50: LC-ESI-MS Analysis of HBD2](image)

**Figure 50: LC-ESI-MS Analysis of HBD2.** A: Ionisation states of purified HBD2. B: High resolution isotope analysis of the 5+ charge state of HBD2. Green circles indicate the predicated mass of fully oxidised HBD2 and red indicated expected mass for the reduced peptide.

Analysis by mass spectrometry indicated that the HBD2 purified was the correct mass (Figure 50) and also fully oxidised. Interestingly, the LC profile of the sample showed two distinct peaks (data not shown), both of which were analysed and had the exact
same mass (therefore not a mix of reduced and oxidised peptides). An explanation for this could be differing disulfide connectivity that would give different HBD2 isoforms that eluted from the column at different retention times.

It might be expected that this method would yield reduced HBD2 as *E. coli* thioredoxin is a cellular oxidoreductase that reduces proteins via cysteine thiol-disulfide exchange. Given the six cysteine residues of HBD2 it could be estimated that Trx interacts with the peptide in some manner to keep the thiols reduced. As it has already been determined that defensins retain their antimicrobial activity after reduction of their disulfide bonds, it is unlikely that this is the reason that the fusion protein is harmless to the cells, and the fact that the peptide is purified in the oxidised form could be attributed to purification under oxidising conditions. Therefore any suggestion that defensins can be expressed as soluble proteins due to the reducing activity of Trx is negated by the discovery that the peptide is purified with disulfide bonds in place. Another plausible possibility is that the defensin is theorised to interact electrostatically with the negatively charged outer membrane of bacterial cells. That Trx-defensin fusions can be expressed in bacteria could be due to both the fusion negating the cationic charge of the defensin and also preventing the secretion of the peptide by virtue of its far greater size. As it stands, the answer to this is still unknown.

Due to the small amount of protein in this sample, the purification was repeated to try and establish disulfide connectivity and ascertain enough protein for NMR analysis. Repeated purification of HBD2, followed by concentration of the peptide by spin filtration, resulted in yields of 720µg HBD2 from 3L of cultured cells. However, MS analysis of these concentrated fractions revealed an interesting artefact in that the peptide had formed dimers. Given that these dimers were observed through ESI-MS it appears that they are covalent in nature, or else would dissociate (Figure 51). Oligomerisation has previously been observed for HBD2 but was thought to be an artefact of high protein concentration in this case (129).

6.2.3 Bioactivity of HBD2 purified from a Thioredoxin-HBD2 fusion.

*As this laboratory lacked the facilities, the bioactivity of the purified peptide was tested in collaboration with Gail Ferguson’s group at the University of Aberdeen.*
HBD2 produced using this methodology was used in bioactivity assays and confirmed that defensin purified in this manner is bioactive against bacteria once it has been cleaved from the fusion partner. The work outlined below has been published (Appendix 8.3).

BacA, is an ABC transporter belonging to *Sinorhizobium meliloti* that is known to play a role in the persistence of the bacterium in symbiosis within legume root nodules of the alfalfa plant *Medicago sativa*. BacA has been shown to confer resistance in the root nodule environment that is rich in host anti-microbial peptides expressed by *M. sativa*. These small, cationic, cysteine rich peptides are similar to the defensin class of AMPs that are expressed in the humans (such as HBD2). A *Mycobacterium tuberculosis* BacA homolog was found to be important for the maintenance of the bacterium in chronic murine infections and was able to partially complement the symbiotic defect of an *S. meliloti* BacA-deficient mutant on alfalfa plants and to protect this mutant *in vitro* from the antimicrobial activity of a synthetic legume peptide, NCR247 and HBD2. Furthermore, *M. tuberculosis* BacA-mediated protection of *S. meliloti* against NC247 and HBD2 is dependent on its attached ATPase domain, which is as expected as the transporter cannot function without ATP hydrolysis. We therefore suggest that *M. tuberculosis* BacA is important for the transport of peptides across the cytoplasmic membrane.
Figure 51: ESI LC FT-ICR MS Analysis of HBD2. A: Mass spectra showing distribution of charge states from concentrated HBD2 samples. Peaks labelled D indicated dimmers. B: Carbon isotope mapping of $[\text{M+5H}]^{+}$ charge state. Green circles indicated expected map for oxidised HBD2, red circles indicate that of the fully reduced form. C: $[\text{m+4H}]^{+}/[2\text{M+8H}]^{+}$ charge states, showing the partial overlap of the two species.
6.3 Conclusions and Further Work.

The first step toward further characterising HBD2 interactions was to develop a method for rapid and efficient production of significant amounts of defensin peptide.

A protocol already described by Vargues et al in our laboratory had achieved production of defensins in a bacterial host cell and 15N-labelled material was prepared for NMR analysis. However, the time consuming and arduous process involved in this methodology was not rewarded in the low yields of oxidised HBD2. This method was also time consuming, required the use of hazardous chemicals, and also needed a fairly skilled protein chemist to carry out the protocol. Therefore it was decided to attempt recombinant production of defensins using another method of expression.

Several methods for purification of soluble defensins have now been published. pET32b thioredoxin systems are commercially available as solubility increasing fusion partners. It has been reported that several antimicrobial peptides have been successfully expressed and purified via a two dimensional chromatography approach. This was an attractive proposition in compared to the insoluble KSI fusion methodology we had already applied.

We designed two constructs, Trx-HBD2 and His-Trx-HBD2. The former was designed to include a TEV protease epitope at the N-terminal of HBD2 and the latter added an N-terminal affinity tag to facilitate better chromatographic purification. Both constructs were expressed and in E. coli BL21 bacteria, providing soluble defensin expression for the first time in our lab. Attempts were made at purification of both fusion proteins with a methodology design based on published pET32b-defensin fusion purifications. However, despite a simple methodology using IMAC to pull down his tagged proteins neither fusion purified as expected.

As Trx-HBD2 could not be purified using IMAC chromatography anion IEX chromatography was used as an enriching step. This had the effect of essentially concentrating the fusion protein in a mixture a little less crude than a cell free extract. As the Trx-HBD2 fusion protein was quite difficult to purify based upon characteristics such as charge (pI 6.3) and size (21664 Da) the HBD2 peptide was cleaved from the fusion at this point. HBD2 is small, cationic and hydrophobic which are theoretically easy to exploit for purification purposes. TEV cleavage was successful, resulting in a
clear ~5kD reduction loss off the fusion peptide and the appearance of a ~5 kDa band when analysed by SDS PAGE (Fig 15).

However, purification of HBD2 from the crude TEV cleavage mixture was unsuccessful. This was attempted using cation IEX chromatography using both a Mono S (SO_3^-) and heparin columns. An additional complication was that TEV protease possesses a polyarginine tag, causing it to elute with HBD2 after cation IEX. As HBD2 has a pI of 9.3 it was expected that the peptide would elute from the cation IEX column at a high salt concentration. However SDS PAGE analysis of the eluted fractions revealed that the peptide had eluted almost immediately. It was concluded that cation IEX chromatography was not an efficient method of purifying cleaved HBD2.

A second thioredoxin-HBD2 construct was then designed in an attempt to improve the first IMAC step. An N-terminal His_6 tag was added to the fusion protein, without deleting the central tag, in the hope that purer Trx-HBD2 fusion could be extracted from cell lysate. After testing various resins, a procedure was developed that did result in purer Trx-HBD2 fusion protein purification, albeit with a few contaminating proteins. Playing on this higher affinity for NiNTA enabled the purification of cleaved HBD2 peptide via a second IMAC step. In this case the peptide was eluted in the flow through.

FT-ICR-MS analysis of these samples revealed that the peptide was purified in a fully oxidised state. HBD2 dimers were observed by mass spectrometry, a phenomenon that is contentious in the literature. Native gel electrophoresis implies that HBD1 & 2 are monomeric, while HBD3 is dimeric (147). Crystallographic data has suggested HBD2 higher order oligomerisation but no NMR analysis has yet seen any form of dimerisation in the absence of glycosaminoglycans (GAG). In this circumstance, dimers were witnessed after concentration of the peptide to ~ 400µM. It maybe that HBD2 dimers are present only in high concentrations of peptide. It has been theorised that oligomerisation is need as a mechanism for killing, and so we may be replicating artificially what happens in a membrane interface.
References


receptor 2


7.1 Analytical s75 Size Exclusion Chromatography Calibration Curve

Figure 52: Calibration curve for analytical s75 Superdex Gel filtration column. 

\[ y = -0.2255x + 1.4642 \]

\[ R^2 = 0.9807 \]

\[ K_{av} = \frac{E_{allution Volume} - 7.46}{24} \]

\[ = \frac{E_{allution Volume}}{\text{Void Volume}} - \frac{7.46}{\text{Bed Volume}} \]

\[ \text{Log MW} \]
7.2 Optically Transparent Thin Layer Cell (OTTLE) Calibration.

The reference electrode was calibrated against Indigotrisulfonic acid (ITS). 1mM ITS was dissolved in 500mM KCl, 10% glycerol, 50mM HEPES pH 7.5. All experiments were performed at 25°C (298 K).

![Graph showing OTTLE Potentiometric titration of Indigotrisulfonic Acid. Differing potentials are applied to the sample and the UV Visible spectrum was obtained on a Cary UV 50 spectrophotometer. Reduced ITS possesses an absorbance maxima at 393nm and the oxidised species at 593nm. The calculated midpoint potential from the experiment was -273 mV. The standard reduction potential of ITS (pH 7.0, 25°C) is -70 mV (Sigma) and so the reference electrode should be corrected by +203 mV.](image-url)
Figure 54: Potentiometric titration of 1mM ITS. The midpoint potential is -273 mV.
7.3 Appendix 3